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PENICILLIN SENSITIVITY OF STRAINS OF SIX COMMON PATHOGENS AND OF HEMOPHILUS HEMOLYTICUS

MANSON MEADS, M.D., EDWIN M. ORY, M.D.,
CLARE WILCOX, AND MAXWELL FINLAND, M.D.
BOSTON, MASS.

SUCCESSFUL penicillin therapy depends mainly on two factors: the susceptibility of the infecting bacteria and the availability of an adequate concentration of the effective agent to act upon the organisms. In general, the most gratifying clinical results have been obtained in those diseases in which the causative organism is susceptible in vitro to concentrations of penicillin which can readily be maintained in the patient. A knowledge of the relative susceptibility of different strains of the common pathogenic organisms, as well as acquaintance with the general range of concentrations to be expected from the use of various doses, intervals, and routes of administration, is, therefore, essential to the intelligent management of penicillin therapy.

In this paper are presented the results of tests for sensitivity to commercial penicillin carried out on a number of strains of common bacteria. Other quantitative aspects of penicillin therapy will be dealt with in separate communications.

MATERIALS AND METHODS

The penicillin sensitivity of the different strains was tested in suitable media by the serial dilution method of Rammelkamp and Maxon[1] modified by the use of a final volume of 1.0 c.c. A strain of hemolytic streptococcus, No. 98, obtained from Dr. C. S. Keefer was used as a standard reference strain throughout this study. Broth cultures containing 1.0 per cent horse blood and seeded with approximately 10,000 of the standard streptococci per cubic centimeter were consistently sterilized by 0.0078 units of commercial penicillin in eighteen hours. Penicillin solutions were prepared at intervals from pools of five vials of commercial penicillin in sterile physiologic saline and stored at 5° C. End points were read as the smallest amounts of penicillin in which there was no evidence of growth at eighteen hours and which yielded no growth in transplants on blood agar after another twenty-four hours of incubation. The crudeness of such a biologic test and its broad limits of error are well recognized.

Most of the strains included in this report were obtained during the course of treatment of various infections on the adult medical wards or during clinical trials of various forms of treatment in cases of acute gonorrhea in the out-patient genitourinary clinic.* Several strains of meningococcus isolated from patients and carriers were obtained through the courtesy of Major Emanuel B. Schoenbach.

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School.

Part of the penicillin used in this study was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

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*The authors are indebted to Dr. Herbert H. Howard for the privilege of treating the patients in this clinic and to Mrs. Helen Trousdale for carrying out the culture studies and for other valuable assistance in the management of these patients.

RESULTS

The susceptibility of a number of strains of each of seven different organisms is shown in Fig. 1 and is indicated both in terms of Oxford units of commercial penicillin and in comparison with streptococcus 98. The variability in the resistance of these organisms and among the different strains of the same organism is at once apparent and has already been noted by Fleming[2] and others.[3-9]

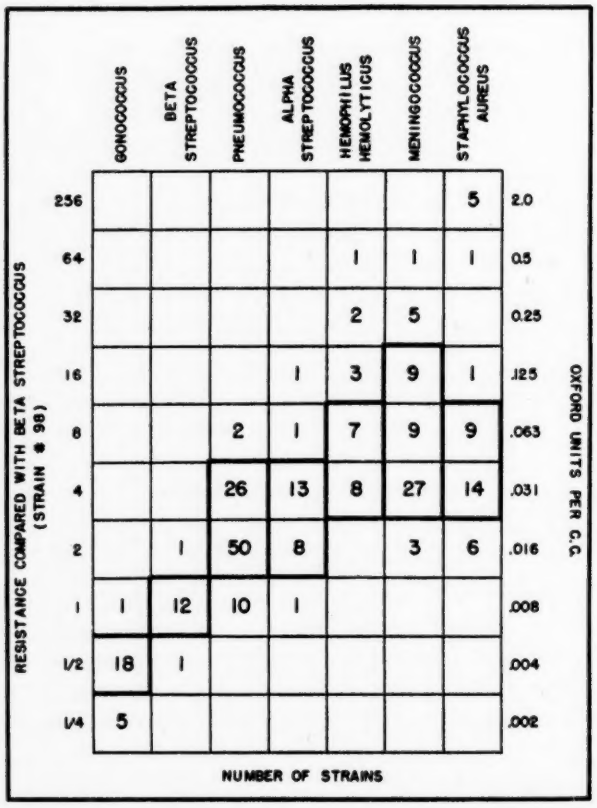

Fig. 1.—Penicillin sensitivity of 261 strains of seven different bacteria compared with the reference strain of hemolytic streptococcus, No. 98.

Gonococcus.—Gonococcus was the most sensitive of the organisms studied. Almost all of the strains were two or four times as sensitive as the reference strain of streptococcus 98. They were all obtained from cultures of cervical or urethral discharge of patients with acute gonorrhea. The majority were sulfonamide resistant, but there was no correlation between the susceptibility to sulfonamides and to penicillin. Similar findings have been reported by others,[10, 11] some of whom have succeeded in increasing the resistance of these organisms in vitro,[12] but naturally resistant strains have not been encountered.

Hemolytic Streptococcus.—Strains of Group A beta hemolytic streptococci were most uniform in their susceptibility to penicillin, almost all of them resembling streptococcus 98 in that respect. The strains listed are chiefly from pharyngeal and blood cultures. A considerably larger number of strains from patients with scarlet fever have also been tested and showed similar sensitivity to penicillin. At least twelve distinct types were represented, but there was no relation between type and penicillin sensitivity among these Group A strains.

Naturally resistant group A strains have not been encountered, but resistance has been produced in vitro[13] and has been found occasionally to increase during therapy.[8] Other Lancefield groups were not studied, but Watson[14] showed that only strains of Group D, *Streptococcus fecalis*, are insensitive, a finding which has been confirmed with respect to strains isolated from urinary tract infections.[15]

Pneumococcus.—The eighty-eight strains of pneumococci represented twenty-four specific types and were isolated chiefly from sputum and blood of patients with pneumonia before treatment. They varied in sensitivity from that equal to the control streptococcus 98 to being eight times more resistant. There was no correlation between the type and the sensitivity of the different strains. Naturally resistant pneumococci have not been encountered. Resistance of pneumococci to penicillin has been increased experimentally without altering their response to sulfonamides[13, 16] or their virulence.[13] Conversely, the development of sulfonamide fastness was found to have no effect on the response to penicillin.[17]

Alpha Streptococcus.—Most of the twenty-four strains of alpha streptococcus listed in Fig. 1 were isolated from the blood of patients with subacute bacterial endocarditis, but a few were obtained in pure culture from infected body fluids or as the predominant organism from nasopharyngeal cultures. Most of them were two or four times more resistant than strain 98. The least sensitive strain was sixteen times more resistant than the control, and the most susceptible one was the same as the control strain. Strains isolated after a course of penicillin therapy in patients with subacute bacterial endocarditis showed differences in cultural characteristics as compared with the original strains, but in one instance a later strain was found to be four times more sensitive than an earlier one.[18] Greater strain variability and the finding of naturally resistant strains have been reported.[2, 6, 19, 20]

Meningococcus.—The fifty-four strains of meningococcus tested were isolated from the blood or spinal fluid of patients with meningitis or from pharyngeal cultures of patients or carriers. They showed a wide range of sensitivity to penicillin, varying from two to sixty-four times more resistant than streptococcus 98. At least two of the more resistant strains were original strains isolated from patients who showed a poor response to treatment with intramuscular and intrathecal penicillin and subsequently responded favorably to sulfonamides.[21] Most of the strains were also tested for sensitivity to sulfonamides and found to be highly susceptible. Serologically, thirty-nine of the strains were classified in Group I, four in Group II, three in Group IIα, and eight could not be classified. There was no correlation between the serologic type and the sensitivity to penicillin.

Staphylococcus aureus.—The thirty-six strains of *Staph. aureus* tested were mostly hemolytic and coagulase-positive strains isolated from the blood or exudates of patients with pneumonia, empyema, arthritis, or acute osteomyelitis. The individual strains showed the widest variation in susceptibility to penicillin, ranging from 2 to 264 times more resistant than the reference strain 98. For at least six of the strains the inhibiting concentrations of penicillin were greater than those which can be readily maintained in the serum during therapy. The occurrence of naturally resistant pathogenic strains of *Staph. aureus* and the development of increased resistance of susceptible strains in vitro or during penicillin therapy have been reported.[1, 4, 7, 9, 13, 22-27] Nine of the strains were nonhemolytic and five were coagulase negative, but there was no correlation between penicillin sensitivity and the hemolytic or coagulase activity.

Hemophilus hemolyticus.—Interest in the susceptibility to penicillin of this apparently nonpathogenic organism was aroused during the course of studies on the effect of a penicillin spray in patients with acute pharyngitis. In some cases in which almost pure cultures of these pleomorphic, gram-negative bacilli were grown from the throat, they were found to disappear rapidly from pharyngeal cultures taken after treatment was begun. The twenty-one strains tested were isolated from throat cultures of patients with acute pharyngitis or from sputum cultures from patients with pneumonia. The range of sensitivity of these strains was found to be similar to that of the great majority of strains of meningococcus and of *Staph. aureus.* They ranged from four to sixty-four times more resistant than streptococcus 98, most of them being only four or eight times more resistant. The only other gram-negative bacillus which has been shown to have a similar sensitivity to penicillin is *Hemophilus ducreyi.*[28] Strains of *Hemophilus influenzae* and *Hemophilus pertussis* have all proved to be highly resistant.[2, 28]

DISCUSSION

The results presented confirm previous reports on the wide variations in susceptibility among organisms generally considered to be sensitive to penicillin as well as among different strains of the same organism. A general acquaintance with the relative sensitivity of the common pathogenic organisms may be of considerable help in the intelligent management of penicillin therapy.[29, 30]

Almost all of the strains of organisms dealt with in this paper have been isolated in relation to penicillin therapy. Except for *H. hemolyticus,* which is included here only because of its peculiar interest as a penicillin-sensitive gram-negative bacillus, these organisms are representative of the great majority of susceptible pathogens encountered in general medical practice. Among these common pathogens only some of the strains of *Staph. aureus* were sufficiently resistant to suggest that they might not yield to systemic therapy as now generally used. Other staphylococcus strains and some strains of meningococcus seem to be sufficiently resistant to require more than average dosage in order to maintain bacteriostatic levels. Other reports suggest that certain strains of alpha or gamma streptococci from patients with subacute bacterial endocarditis likewise require relatively high concentrations of penicillin for the optimum effect.

Meningococci, fortunately, are quite uniformly sensitive to sulfonamides. Penicillin-resistant staphylococci and streptococci, on the other hand, still pose a challenging therapeutic problem because the focal lesions which they produce may be inaccessible for topical use of the antibiotic in sufficient concentration. In addition, these organisms are relatively resistant to sulfonamides as well.

The contrast between the gonococcus and the meningococcus is of some interest. Both are usually considered to be sensitive to penicillin and to sulfonamides. The gonococcus, however, appears to be considerably more susceptible to penicillin, while the meningococcus seems to be much more sensitive to sulfonamides. Sulfonamide resistance is apparently readily induced during treatment of gonococcal infections. At any rate, sulfonamide-resistant strains of gonococci are being encountered with increasing frequency, whereas such resistance has not been encountered among meningococci. The reverse, however, seems to be true with respect to penicillin—relatively resistant strains are encountered among meningococci but not among gonococci.

The exact nature of penicillin resistance is still a matter for speculation. The majority, though not all, of the naturally resistant organisms have been

found to contain or to produce a penicillin-inactivating enzyme.[24, 31-33] Development of penicillin resistance, however, was found not to be associated with the acquisition by the bacterium of the ability to produce such a penicillinase.[24] There is some evidence that the development of resistance by bacteria exposed to proper concentrations of penicillin may be a process of selection by which susceptible individuals are gradually eliminated as the concentration of the antibiotic is increased. This leaves the more resistant individuals which then reproduce further generations of resistant individuals.[26]

SUMMARY

Tests for sensitivity to commercial penicillin in vitro have been carried out on 240 pathogenic strains of gonococcus, beta and alpha streptococcus, pneumococcus, meningococcus, and staphylococcus and also on twenty-one respiratory strains of *H. hemolyticus*. There were wide differences in sensitivity among these organisms and among different strains of the same organism.

The strains of gonococcus and of Group A hemolytic streptococcus were the most sensitive to penicillin and showed the greatest uniformity in that respect. The strains of staphylococcus and meningococcus showed the widest range of sensitivity. Most of the *H. hemolyticus* strains reacted like the majority of staphylococci and meningococci. The sensitivity of strains of pneumococcus and alpha streptococcus was intermediate between that of beta streptococci and meningococci.

Resistant strains, which were 256 times more resistant than the reference strain of hemolytic streptococcus, were encountered only among the staphylococci. Relatively insensitive strains, which were from sixteen to sixty-four times more resistant than the reference strain, were found among the staphylococci, meningococci, *H. hemolyticus,* and alpha streptococci.

Among the Group A streptococci, pneumococci, and meningococci there was no correlation between the serologic types and penicillin sensitivity. Among the pathogenic staphylococci there was no apparent relation between penicillin sensitivity and the hemolytic or coagulase properties of the different strains.

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THE SUPERIORITY OF THE CONTINUOUS INTRAVENOUS DRIP FOR THE MAINTENANCE OF EFFECTUAL SERUM LEVELS OF PENICILLIN: COMPARATIVE STUDIES WITH PARTICULAR REFERENCE TO FRACTIONAL AND CONTINUOUS INTRAMUSCULAR ADMINISTRATION

LEO LOEWE, M.D., PHILIP ROSENBLATT, M.D., MORTIMER RUSSELL, AND ERNA ALTURE-WERBER, PH.D.
BROOKLYN, N. Y.

THE treatment of infections with penicillin has been largely empiric, and dosage schedules generally have been evolved by trial and error. Great stress has been placed upon the sensitivities to penicillin of various organisms which have been classified as sensitive or insensitive. Few assays have been made upon body fluids in order to determine whether effective penicillin levels have been obtained and whether they were being maintained.

From the Department of Medicine and the Department of Laboratories, Jewish Hospital of Brooklyn.

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The treatment of certain diseases such as gonorrhea is relatively simple because of the extreme sensitivity of the infecting organism to small doses of penicillin. The treatment of diseases such as thrombo-ulcerative endocarditis due to alpha or gamma streptococci is fraught with difficulties because of the great variability in the sensitivity of the infecting organisms and the need for prolonged adequate dosage. It is highly desirable to be able to predict, if only approximately, what blood penicillin levels may be obtained and how long they will be sustained with a given technique of administration. Studies of this type have been limited. Rammelkamp and Keefer[1] have shown that the intravenous injection of a single dose of penicillin resulted in a very rapid rise and a rapid fall of the blood level so that at the end of two hours no detectable amounts remained. Absorption was somewhat slower and blood levels were better maintained following intramuscular administration. The subcutaneous administration was followed by irregular absorption and low blood levels. Cooke and Goldring[2] have demonstrated similar findings in children treated with penicillin.

Rantz and Kirby[3] have reported on the absorption and excretion of penicillin following continuous intravenous and subcutaneous administration. They find an approximately linear relationship between plasma penicillin levels and the rate of intravenous penicillin infusion when the material is given at a constant rate of flow. The continuous subcutaneous method was associated with plasma levels much lower than those following the intravenous method. The dosage schedules used did not exceed 480,000 units per day; clearance studies indicated that the maximal tubular excretory capacity of the kidneys had not been reached with this amount.

METHODS AND MATERIALS

Penicillin: Penicillin was originally supplied as the sodium salt, powdered, dehydrated, and in vacuo, in bottles each containing 100,000 units. Appropriate amounts were dissolved in sterile, pyrogen-free isotonic saline or Ringer's solution. With massive dosages, up to 10,000,000 units daily, the solution of so much dry powder proved arduous and entailed considerable loss so that sterile solutions* of sodium penicillin were especially prepared in a concentration of 100,000 units per cubic centimeter.

Penicillin Assay: Serum and urine were titrated routinely for penicillin content by the microbiologic method of Rosenblatt, Alture-Werber, Kashdan, and Loewe.[4]

Blood: Blood was collected aseptically by syringe and transferred immediately to a sterile test tube. This was allowed to clot in the refrigerator and the separated serum was assayed for its penicillin content.

Administration: When given by continuous intravenous drip,[5-8] suitable amounts of penicillin were dissolved in 1,000 c.c. of either isotonic saline or Ringer's solution. This was allocated over a twenty-four hour period. With intramuscular dosage, the appropriate amount of penicillin was dissolved in 2 c.c. of isotonic saline or 1 per cent novocain solution. The anesthetic vehicle was used if the subjects complained excessively of local pain. It was previously determined that 1 per cent novocain solution had no antibiotic influence.

Subjects: The subjects were all adult patients under treatment for subacute bacterial endocarditis.[9-11] The sexes were approximately equally repre-

*We are extremely indebted to Mr. John L. Smith, of the Charles Pfizer Company, for his keen interest, valuable suggestions, and constant cooperation. Through him we were enabled to obtain the lavish supplies of penicillin utilized in all our experimental studies. The especially prepared solution of penicillin proved to be convenient, time saving, and stable at refrigerator temperature over a period of at least two weeks.

sented. No attempt was made to correlate body weight, habitus, or temperature with dosage or blood levels.

Serum Penicillin Concentrations Following Fractional Intramuscular Dosage.—The most popular mode of penicillin administration is fractional intramuscular injection. In Table I is a compilation of average blood levels obtained in several hundred determinations done on approximately 100 different subjects. It must be emphasized that these are average curves for given dosage schedules. Individual determinations on the same or different subjects may vary from the norm by as much as 25 to 100 per cent.

TABLE I. PENICILLIN SERUM LEVELS FOLLOWING FRACTIONAL INTRAMUSCULAR ADMINISTRATION

PENICILLIN DOSAGE (OXFORD UNITS)		SERUM TITER (OXFORD UNITS PER C.C.)			
INDIVIDUAL DOSE	EQUIVALENT DAILY DOSE (EVERY 2 HR.)	30 MINUTES	60 MINUTES	90 MINUTES	120 MINUTES
10,000	120,000	0.15	0.06	–	–
16,666	200,000	0.15	0.06	0.01	–
20,000	240,000	0.2	0.09	0.035	0.01
25,000	300,000	0.35	0.2	0.063	0.03
40,000	480,000	0.5	0.33	0.22	0.09
41,666	500,000	0.7	0.45	0.25	0.15
50,000	600,000	1.0	0.6	0.3	0.2
83,333	1,000,000	1.5	0.75	0.4	0.25
100,000	1,200,000	1.7	0.8	0.625	0.3

Results: Maximum serum levels are obtained within thirty minutes. At the end of one hour, levels fall sharply to approximate 50 per cent of the thirty-minute figure. Thereafter the drop is less precipitate, but at the end of an hour, significant amounts of detectable penicillin are not present in the serum except with high dosage.

Comment: When the sensitivity of an infecting organism is known, it is a simple matter to choose the proper dosage schedule. For example, most strains of beta hemolytic streptococci are highly susceptible to penicillin and are inhibited in vitro in concentrations of from 0.008 to 0.015 unit per cubic centimeter. Dosages of penicillin between 20,000 and 25,000 units administered every two hours should satisfactorily inhibit this type of organism.

The relatively resistant organisms such as the alpha and gamma streptococci, usually found in thrombo-ulcerative endocarditis, present a more difficult problem. Many strains in these groups have a penicillin sensitivity of from 0.15 to 0.6 unit per cubic centimeter or higher. With intramuscular dosage the ninety-minute or two-hour serum penicillin level is almost always below the in vitro sensitivity of the organism. Thus, in effect, therapy may be ineffectual for a significant portion of the time between injections. Situations of this type are hazardous, for there is evidence to prove that inadequate dosage may result in treatment failure due to acquired increased resistance[12] of organisms to the action of penicillin.

Many patients do not tolerate fractional intramuscular injections despite the fact that different sites are chosen for injection. Local pain may be alleviated by using a solution of novocain as a vehicle, but this is not suitable for long courses of therapy. Furthermore, the necessity for frequently repeated injections interferes with the rest and morale of the patient.

Briefly, in the treatment of disease due to bacteria which are highly susceptible to the action of penicillin and where the span of therapy is relatively short (for example, gonorrhea, pneumonia), the fractional intramuscular method of penicillin administration can be recommended.

Serum Penicillin Concentrations Following Continuous Intramuscular Drip.—The administration of penicillin by continuous intramuscular infusion has been recommended by Harris[13] and by Dawson and Hunter.[14] The latter authors report that this method of administration appears to be the procedure of choice for patients receiving penicillin for prolonged periods. They state that comparative studies usually show that higher blood levels are obtained by continuous intramuscular drip than by continuous intravenous drip. They consider intramuscular drip technically simpler and better tolerated by the patient.

Experimental: Comparative levels were determined in a number of patients. The daily dosage of penicillin varied from 500,000 to 5,000,000 Oxford units dissolved in 750 c.c. of sterile isotonic saline solution. At the end of each day, blood was drawn for determination of the serum penicillin content.

TABLE II. PENICILLIN SERUM LEVELS FOLLOWING CONTINUOUS INTRAMUSCULAR ADMINISTRATION

DAILY PENICILLIN IN OXFORD UNITS (X 1,000)	AVERAGE SERUM LEVEL UNITS PER C.C.
500	0.35
1,000	0.6
2,000	1.5
3,000	2.0
5,000	5.0

Results: The average serum penicillin levels are given in Table II. Individual determinations varied as much as 100 per cent from the norm. In the same patient, under identical experimental conditions, serum penicillin concentrations, following the continuous intramuscular drip, tended to be consistently lower than those obtained by the continuous intravenous drip (Fig. 1).

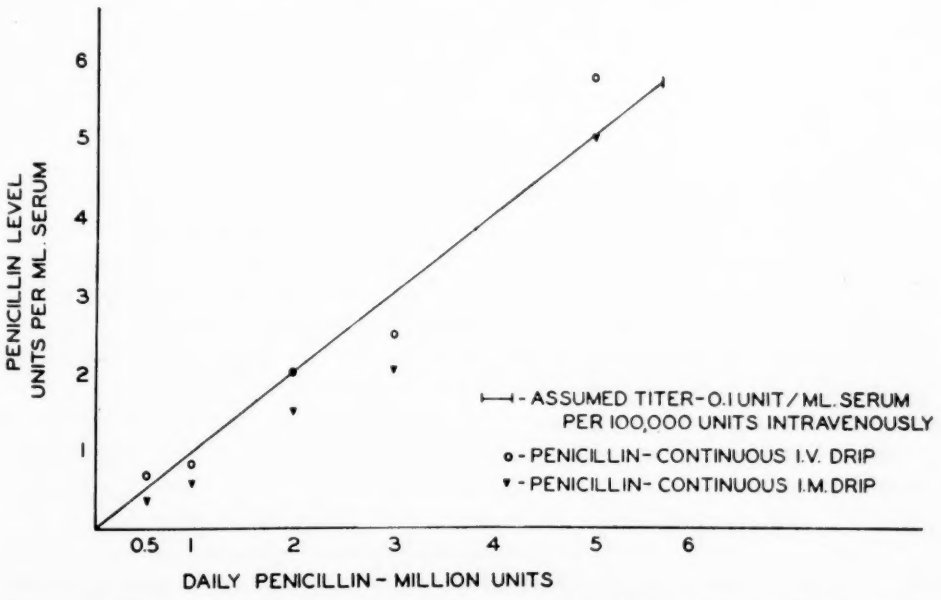

Fig. 1.—Comparison of serum penicillin levels following continuous intramuscular and continuous intravenous infusions.

Comment: The method of continuous intramuscular infusion is technically simple in so far as its administration is concerned. However, its maintenance, without serious complaint from the patient, is another matter. In our experience, the thigh muscles of the average patient tolerated only 750 c.c. of solution

per day. More than this amount almost invariably resulted in acute muscular soreness, with or without accompanying fever, and necessitated discontinuance of the infusion despite the fact that the site of the drip was changed from one thigh to the other each day. It was almost impossible to regulate the flow of less than 750 c.c. daily in a satisfactory manner.

Serum Penicillin Concentrations Following Continuous Intravenous Drip.—Rantz and Kirby[3] have shown that when penicillin is administered, at a constant rate, by continuous intravenous drip and in dosages up to 20,000 units per hour (480,000 units per day), predictable plasma levels can be obtained approximating 0.1 unit per cubic centimeter of plasma per 100,000 units of penicillin per day. We have extended these studies to include penicillin serum concentrations following continuous intravenous infusion in dosage up to 10,000,000 units per day.

Results: The results of serum penicillin assays are noted in Table III. In general, the values obtained are consistent with those anticipated, that is, 0.1 unit per cubic centimeter serum for each 100,000 units per day. It should be noted, however, that there is considerable variation in individual levels on different patients and even in the same patient at different times. These variations are due to fluctuations in the rate of flow of the intravenous solution and to mutations in the physiologic activity of the patients from time to time.

TABLE III. PENICILLIN SERUM LEVELS FOLLOWING CONTINUOUS INTRAVENOUS INFUSION

DAILY PENICILLIN IN OXFORD UNITS (x 1,000)	AVERAGE SERUM LEVEL UNITS PER C.C.	EXPECTED LEVEL UNITS PER C.C.	PER CENT DEVIATION FROM EXPECTED LEVEL
200	0.20	0.20	0.0
250	0.27	0.25	+ 8.0
300	0.34	0.30	+13.3
500	0.57	0.50	+14.0
900	0.90	0.90	0.0
1,000	0.91	1.0	- 9.0
2,000	2.0	2.0	0.0
3,000	2.6	3.0	-13.3
5,000	5.8	5.0	+16.0
10,000	9.2	10.0	- 8.0

Comment: The method of continuous intravenous drip is technically more difficult than fractional or continuous intramuscular dosage. However, the use of 23-gauge needles and the utilization of veins about the wrist, forearm, and lower extremities circumvent many inherent disadvantages. The method is attended with minimum discomfort to the patient and is extremely well tolerated. The incorporation of heparin in the venoclysis or its subcutaneous[15] deposition are valuable adjuncts for continuous intravenous therapy.

With meticulous attention to detail and the conjoint use of heparin, it is possible to keep the drip at a single site for as long as fourteen days. It is preferable, however, to change the site every three or four days to prevent undesirable local irritation, endophlebitis, or an occasional pyrogenic reaction.

The high serum levels make it possible to attack infections heretofore considered inaccessible to penicillin therapy. The combination of massive dosage schedules with such potentiating substances as para-aminohippuric acid[16] may yield even higher serum levels. Extreme resistance of an infecting agent should no longer be a deterrent to therapy; rather it should be a stimulus to vigorous, intensive attack.

It is significant to note that massive dosage up to 10,000,000 units per day is not attended with any symptoms of toxicity, either immediate or delayed.

Patients have been observed for months following massive dosage without any evidence of tissue damage.

CONCLUSIONS

1. Comparative studies of fractional intramuscular, continuous intramuscular, and continuous intravenous administration of penicillin have been presented.

2. Fractional intramuscular therapy is recommended for the treatment of acute, short-lived infections. Dosages should be given every two hours.

3. The continuous intravenous drip of penicillin yields serum levels superior to those obtained following continuous intramuscular injection.

4. Predictable serum penicillin levels are obtained following continuous intravenous drip in daily dosage schedules up to 10,000,000 units.

5. Daily dosages of penicillin up to 10,000,000 units are nontoxic.

6. The relation of these observations to the treatment of infections is indicated.

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THE ACTIVITY OF PENICILLIN AT TEMPERATURES ABOVE 37° C.

ROBERT E. HOYT, PH.D., O. B. PRATT, M.D., AND MILTON G. LEVINE, PH.D.
LOS ANGELES, CALIF.

IT IS well known that compounds which inhibit bacterial growth exhibit an increase in efficiency as the temperature of incubation is raised. Eagle and Musselman* have shown that the spirocheticidal effect of penicillin is enhanced by increasing temperatures. That this relationship holds for the action of penicillin upon *Staphylococcus aureus* is shown by the following observations.

Varying amounts of calcium penicillin were distributed in matched colorimeter tubes, and sufficient broth was added to bring the volume up to 1 c.c. The inoculum consisted of 4 c.c. of dextrose broth seeded with an eighteen-hour culture of *Staph. aureus*. The tubes were read in a Klett-Summerson photelometer before and after incubation, the difference between the two values being taken to represent the relative bacterial growths. The period of incubation necessary to produce a suitable turbidity was related to the size of the inoculum, 1 c.c. of culture added to 100 c.c. broth giving reliable readings in three hours. Use of a smaller inoculum extended the incubation period to five or six hours but did not influence the results of the test. The observations have been repeated six times. On each occasion duplicate titration curves were prepared for each of the temperatures tested. While the differences in activity were not always of the same magnitude, the order was always the same, the penicillin showing greater activity at the higher temperature. Typical data are recorded in Tables I and II. The results are shown graphically in Fig. 1.

TABLE I. 100 C.C. BROTH SEEDED WITH 1 C.C. EIGHTEEN-HOUR CULTURE OF STAPH. AUREUS; INCUBATION PERIOD, THREE HOURS

PENICILLIN		BROTH	INOCULUM	TURBIDITY	
O. U.	VOLUME (C.C.)	(C.C.)	(C.C.)	37° C.	41° C.
0	0	1	4	85	82
0.02	0.2	0.8	4	85	58
0.04	0.4	0.6	4	65	24
0.06	0.6	0.4	4	44	14
0.08	0.8	0.2	4	28	7
0.10	0.1	0	4	7	7

TABLE II. 100 C.C. BROTH SEEDED WITH 0.1 C.C. EIGHTEEN-HOUR CULTURE OF STAPHYLOCOCCUS AUREUS, INCUBATION PERIOD, SIX HOURS

PENICILLIN		BROTH	INOCULUM	TURBIDITY		
O. U.	VOLUME (C.C.)	(C.C.)	(C.C.)	37° C.	39° C.	41° C.
0	0	1	4	100	93	44
0.005	0.25	0.75	4	96	86	39
0.01	0.5	0.5	4	98	79	28
0.02	1.0	0	4	83	58	18

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*Eagle, Harry, and Musselman, A. D.: The Spirocheticidal Action of Penicillin in Vitro and Its Temperature Coefficient, J. Exper. Med. **80**: 493-505, 1944.

It is evident that incubation at 39 and 41° C. increases the activity of the penicillin as detected by turbidity measurement. In the series which received the smaller inoculum the growth at 41° C. was less than that obtained at lower temperatures, but when the relative inhibition is calculated, using the control tube as 100 per cent, it is seen that the order compares well with the sets showing heavier growth.

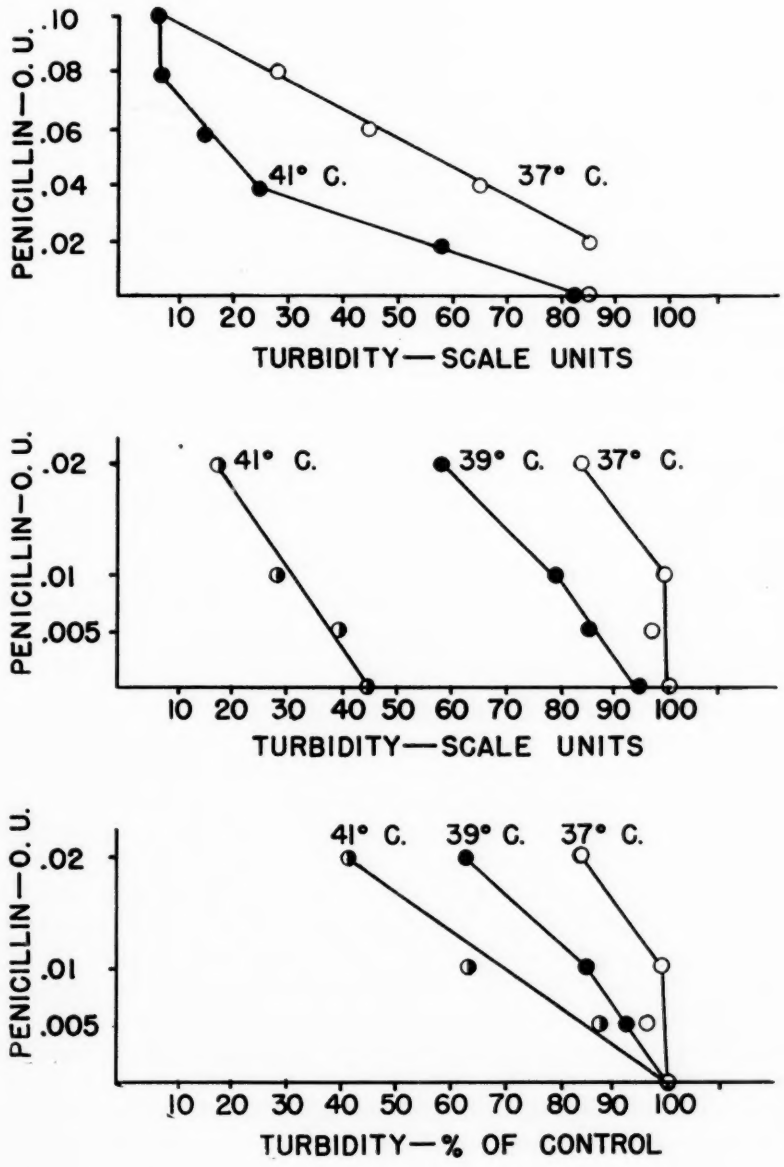

Fig. 1.

Two significant conclusions can be drawn from these facts. The combination of penicillin and fever therapy, suggested by Eagle and Musselman for the treatment of syphilis, may be expected to apply to other infections where penicillin alone is of value. A second application is in the estimation of penicillin levels using the measurement of turbidity as an index. By carrying out the incubation at 41° C., values of 0.005 Oxford unit can be detected with increased precision and accuracy, using *Staph. aureus* as the test organism.

ORAL PENICILLIN IN THE TREATMENT OF GONORRHEA

ALFRED H. FREE, PH.D., LYMAN F. HUFFMAN, M.D., HARRY R. TRATTNER, M.D., AND HELEN B. BROWN, PH.D.
CLEVELAND, OHIO

IT HAS recently been demonstrated by Free, Leonards, McCullagh, and Biro[1] that orally ingested penicillin is absorbed from the gastrointestinal tract in amounts that would be therapeutically effective. This finding has since been confirmed by several laboratories,[2-7] and obviously indicates the desirability of determining the effectiveness of orally ingested penicillin in the treatment of infections.

It has been shown that gonorrhea is particularly susceptible to treatment with penicillin,[8] a finding that has been confirmed by numerous clinical studies. Several recent reports have indicated that in uncomplicated gonorrhea better than 99 per cent of the cases responded favorably to treatment with penicillin.[9-12]

The present report describes studies carried out in a small series of patients with gonorrhea who were treated with orally administered penicillin.

METHODS

All of the patients in the series were diagnosed as having gonorrhea by means of positive cultures and by clinical signs, symptoms, and history. The penicillin used was in the form of a dry powder contained in gelatin sleeve capsules. Each patient ingested a total of 1.6 million units of penicillin over a two-day period. The dosage schedule involved taking 100,000 units every two hours during the waking period. In all cases the patient was given the requisite amount of penicillin along with explicit instructions regarding the time and number of capsules to be ingested. The first eight patients indicated in Table I were not hospitalized, whereas Patients 9 through 14 were all treated during hospitalization. A complete forty-eight-hour urine sample was collected during the period of therapy, and the total penicillin excretion was determined by assaying the urine, using the cylinder plate method of Schmidt and Moyer.[13] Two urethral cultures in the male patients and two urethral and two cervical cultures in the female patients were obtained during the ten-day period following treatment, and in most of the patients a third culture was obtained at a somewhat later time.

RESULTS

A total of fourteen patients with gonorrhea have been treated by the technique described. In all instances only negative cultures were obtained following treatment and all clinical signs and symptoms of gonorrhea disappeared. Table I shows the sex distribution of the patients and also presents the data for the urinary excretion of penicillin. The average excretion represents approximately 6 per cent of the ingested penicillin.

From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, the private practice of Dr. Huffman, Cleveland, the Urological Service of Cleveland City Hospital, and Ben Venue Laboratories, Bedford, Ohio.
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No toxic effects were observed with this quantity of orally ingested penicillin, although one of the patients did complain of a certain amount of gastric distress and diarrhea. This effect could readily be due to the state of purity of the penicillin used in these studies since it was of a somewhat lower potency than most of the penicillin that is used for parenteral therapy. In all of the patients there was rapid improvement soon after therapy was begun and cultures taken three months after treatment in the first eight patients were all negative. In none of these patients has there been any recurrence of clinical signs or symptoms over a six-month period.

TABLE I

PATIENT	SEX	URINARY EXCRETION OF PENICILLIN (TOTAL OXFORD UNITS EXCRETED)	CLINICAL RESULT
1	M	92,000	Cure; 3 negative cultures after treatment
2	F	87,000	Cure; 3 negative cultures after treatment
3	M	110,000	Cure; 3 negative cultures after treatment
4	M	111,000	Cure; 3 negative cultures after treatment
5	M	166,000	Cure; 3 negative cultures after treatment
6	M	35,200	Cure; 3 negative cultures after treatment
7	M	63,000	Cure; 3 negative cultures after treatment
8	M	31,500	Cure; 3 negative cultures after treatment
9	F	112,500	Cure; 1 negative culture after treatment
10	F	74,000	Cure; 3 negative cultures after treatment
11	F	68,000	Cure; 2 negative cultures after treatment
12	F	70,000	Cure; 2 negative cultures after treatment
13	F	60,000	Cure; 2 negative cultures after treatment
14	F	59,000	Cure; 2 negative cultures after treatment

DISCUSSION

In the present study the amount of penicillin employed was quite large but the results were uniformly successful. There is no reason to believe that this amount is necessarily required for successful treatment of gonorrhea, and there are considerations that indicate that a considerably smaller quantity would be effective. For instance, it has been found that with parenteral therapy 100 per cent successful results were obtained in 865 patients with gonococcal infection of the urethra who were treated with a total of 75,000 units given over a one-day period.[12] In half of our patients the amount of penicillin excreted in the urine was in excess of this amount. Certainly all of the penicillin that can be observed in the urine has been absorbed from the gastrointestinal tract. At the present time there is some indication that the amount of intestinal absorption can be increased by various means.

Since the completion of this work, a report has appeared by György and associates,[14] which describes the successful treatment of gonorrhea with oral penicillin. The findings of the present study offer confirmation of this observation.

Even though the quantity of penicillin required to treat gonorrhea and presumably other infections may be greater if the penicillin is given orally than if it is given parenterally, the convenience to the patient and the physician is such that with ample supplies of the drug available this appears to outweigh the added cost of the drug itself. This is especially true since for most parenteral therapy with penicillin it has been almost essential that the patient be hospitalized during the course of treatment. The use of various techniques to decrease the absorption of penicillin from the site of intramuscular injection

or to decrease the rate of urinary excretion of penicillin may obviate the need of hospitalization. However, the advantages of orally administered penicillin over these various other forms are quite apparent. The conditions of treatment employed with eight patients in the present study did not involve hospitalization and in these cases the patient took the medication merely following the instructions of the physician.

SUMMARY

Fourteen patients with gonorrhea, both men and women, have been successfully treated with penicillin administered by mouth. In no instance was the method of treatment unsuccessful. The advantages of orally administered penicillin in therapy are discussed.

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POTENTIATION OF GERMICIDES WITH AEROSOL OT

WALTER C. TOBIE, PH.D., AND MARTHA L. ORR, B.S.
STAMFORD, CONN.

THE action of surface tension depressants in increasing (potentiating or synergizing) the activity of germicides and fungicides has received considerable attention in recent years.[5-10, 12, 13, 15] We have previously briefly noted the action of Aerosol OT (di-2-ethylhexyl sodium sulfosuccinate) in this respect.[14] The present paper gives quantitative data and deals with some of the factors involved in this action.

METHODS AND MATERIALS

The method of the Food and Drug Administration known as "the F. D. A. method (special), *Staphylococcus aureus*, 37° C.,"[1, 2] was closely followed except for the use of sterile 0.2 c.c. Kahn pipettes to take 0.02 c.c. of medication mixture for inoculations. The reason for the use of the pipettes is that surface tension depressants such as Aerosol OT reduce the size of the drop (inoculum) taken on the standard loop from 0.02 c.c. to a much smaller volume, giving irregular results and making the potentiation appear to be greater than is actually the case. Details are given in our previous note.[14]

The culture of *Staph. aureus* used was No. 26 of our collection, which is No. 209 of the F. D. A. collection. Aerosol OT, when used, was always present in a concentration of 0.1 per cent in the medication tubes, a 1.0 per cent solution in distilled water being used in making up the dilutions. Concentrations are expressed in terms of grams of substance per 100 c.c. of solution. If allowed to stand for a day or so before use, the 1.0 per cent working solutions of Aerosol OT gave no bacterial growth when inoculated into the so-called "Reddish broth" used in cultivating the test organism in the F. D. A. method. Accordingly, no effort was made to sterilize the Aerosol OT working solution. This self-sterilizing action of 1 per cent Aerosol OT has been noted previously.[9]

The various dilutions of phenol were made from a solution of the pure compound standardized at 5.00 per cent by the method of the U. S. XI.[3, p. 285]

The cresol was Merck's U. S. P. grade. According to Merck's Index,[4] this is a mixture of ortho-, meta-, and para-cresols (in proportions not stated) and usually contains a few per cent of phenol. Although it is not a chemical individual but a mixture of compounds, it is nevertheless one of the purer forms of cresol commonly used in medicine and pharmacy. Since its density is almost exactly the same as that of water, the 1.00 per cent solution used in making the dilutions was prepared by pipetting exactly 2.00 c.c. into a 200 c.c. volumetric flask and making to volume with distilled water. The brown drops of cresol dissolved rather slowly to a clear solution. Although cresol is said to be soluble in about 50 parts of water,[4] attempts to prepare 2 per cent solutions were not successful, since a portion of the cresol persistently refused to dissolve.

The iodine was used in the form of tincture of iodine (strong), prepared in our laboratory according to the specifications of U. S. P. XI, containing 70

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From the Research Laboratories of the American Cyanamid Co.

Gm. of iodine, 50 Gm. of KI, 50 c.c. of water, and made to 1 liter with 95 per cent ethyl alcohol. In making the dilutions in the medication tubes, a 1 per cent by volume aqueous solution of the tincture of iodine was used. In reporting the final results in terms of phenol coefficient, the limiting dilutions were recalculated into terms of concentrations *by weight* of tincture of iodine. Since the tincture had a specific gravity of exactly 0.900, the recomputation was easily done, although it would have been preferable to have made up the 1 per cent solution by weight rather than by volume.

The phenylmercuric nitrate was from Eastman Kodak Co., No. 3240. Exactly 1.000 Gm. was placed in a 2 liter volumetric flask and made nearly to volume. On heating and agitation in a water bath, the compound dissolved slowly. The solution was cooled to room temperature, then made to volume, giving a $\frac{1}{2000}$ (0.05 per cent) working solution.

RESULTS

Each phenol coefficient was run at least four times, both with and without Aerosol OT. It will be seen from Table I that 0.1 per cent Aerosol OT increases the germicidal action of phenol and cresol about 1.8 times (80 per cent increase). This increase was fairly uniform in different runs, which checked rather well with each other.

TABLE I. INCREASE IN PHENOL COEFFICIENTS OF GERMICIDES AGAINST STAPH. AUREUS AT 37° C. IN PRESENCE OF 0.1 PER CENT AEROSOL OT

GERMICIDE	PHENOL COEFFICIENTS		INCREASE IN PHENOL COEFFICIENT (OR BACTERIOSTATIC POWER)	
	GERMICIDE ALONE	GERMICIDE IN PRESENCE OF 0.1% AEROSOL OT	RATIO	PER CENT INCREASE
Phenol	1.0	1.8	1.80	80
Cresol, U. S. P.	2.4	4.4	1.83	83
Tincture of iodine (strong)	3.7-4.3	4.3-5.5	1.0-1.5	0-50
Phenylmercuric nitrate	166*	1300*	8.0	685

*Bacteriostatic power only. True bactericidal power not determined.

With tincture of iodine, the results of different runs were very variable, whether Aerosol OT was used or not. It potentiated the germicidal action only slightly and irregularly, from no action at all to a 50 per cent increase when the lowest values for iodine are compared with the highest results for iodine plus Aerosol OT. When the iodine was added to the medication tubes, its brown color was partly discharged, undoubtedly because of chemical reaction with the constituents of the broth. This is in line with the well-known fact[1] that the phenol coefficient method is not particularly satisfactory when applied to preparations containing active halogen. It is also analogous to the findings of Gershenfeld and co-workers,[9] that Aerosol OT has little or no action in potentiating the germicidal action of hypochlorite (Zonite).

The most striking results were obtained with phenylmercuric nitrate. Although the results in different runs varied somewhat, it was nevertheless possible to determine fair average values, in which it was found that Aerosol OT increased the bacteriostatic power eightfold. It was not determined whether this represented an increase in true killing power or in bacteriostatic action only. It is possible that this great increase is due to the formation of a new compound in the solution, namely phenylmercuric di-2-ethylhexyl sulfosuccinate, since when 1 per cent solutions of Aerosol OT are mixed with 0.05 per cent solu-

tions of phenylmercuric nitrate in equimolecular amounts, a strong haze appears. Attempts to prepare such a compound in the dry state led to disappointing results, since the compound (if formed) appeared to decompose on drying. No further work is planned, but it seems probable that compounds of the phenylmercuric radical with the negative radical of appropriate surface active compounds might give germicidal or bacteriostatic compounds of great potency.

DIFFICULTIES IN THE F. D. A. PHENOL COEFFICIENT DETERMINATION

The usual difficulties connected with the current method of phenol coefficient determination were encountered. One of these was a reduced resistance of *S. aureus* to phenol. The limiting dilution (which would kill in ten minutes but not in five minutes at 37° C.) was frequently not 1-80, but 1-85 or 1-90. When this occurred, it was assumed that the resistance to other germicides diminished in essentially the same ratio, and the phenol coefficient was calculated under the conditions prevailing. Another difficulty was the familiar one of "skips," a higher dilution of germicide killing the test organism, while the next lower dilution failed to do so. This trouble was more common with iodine and phenylmercuric nitrate than with phenol and cresol. A discussion of the causes of these difficulties and irregularities must be left for a future paper.

CHARACTERISTICS OF AEROSOL OT

Reduction of Surface Tension.—To determine the reduction of surface tension by Aerosol OT, readings were taken with the DuNouy tensiometer. The following data represent the average of four determinations in each case, in terms of dynes: distilled water, 70.7; medication tube mixture, 1-90 phenol, 51.1; medication tube mixture, 1-100 phenol, 53.3, which diminished to 29.8 with 0.1 per cent Aerosol OT present. The 1.0 per cent Aerosol OT working solution had a value of 28.9 dynes. The potentiating action of Aerosol OT is presumably due, at least in part, to a reduction of the interfacial tension between the bacterial cells and the surrounding liquid, so that the germicide reaches or penetrates the cells more readily.

Acidity of Old Aerosol OT Solutions.—Solutions of Aerosol OT gradually become more acid with age. When freshly prepared, such solutions have a pH of from 6.3 to 6.9, closely approaching that of the distilled water used, since Aerosol OT has little or no buffering power. However, the solutions showed very little decrease in pH at first. One 5 per cent solution stored in the refrigerator for twenty-two days showed a pH of 6.90 by the glass electrode. Older solutions showed lower pH values. Of those held at room temperature, one at 4.47 per cent had a pH of 2.90 after sixteen months, while a 0.04 per cent solution had a pH of 5.79 after fifteen months. The acidity of these old solutions is undoubtedly due to partial hydrolysis of the Aerosol OT with liberation of acid. Aerosol OT solutions are unstable in the presence of any great amount of electrolyte, the Aerosol OT settling out in the form of droplets. Accordingly, efforts to buffer solutions of Aerosol OT at pH 7 were unsuccessful. The procedure of using reasonably fresh 1.0 per cent solutions of Aerosol OT, with a pH of not less than 6.30, was adopted. Due to the poor buffering power of Aerosol OT, such solutions gave no appreciable shift in pH when added to the liquid in the medication tubes, so that the potentiating action was not due to any diminution in pH but only to the reduction of interfacial tension between the liquid and the cells or to other factors.

Increased Potentiation by Old (Acidic) Aerosol OT Solutions.—In our earlier work, before the pH of the Aerosol OT solutions was properly controlled,

a somewhat greater potentiating action was obtained than with the nearly neutral solutions used later. A 1.0 per cent solution (prepared from the sixteen-month-old 4.47 per cent solution at pH 2.90) had a pH of 2.88, depressing the pH of medication tube mixtures containing phenol from 6.8 to about 5.2 to 5.4, while increasing the apparent phenol coefficient of the phenol by 100 per cent instead of 80 per cent. This increase in the activity of germicides with diminished pH is well known[9, 11, 12] and must always be taken into account in potentiation experiments. In certain applications it would be preferable to use potentiated germicide solutions at the lowest possible pH.

SUMMARY

Under conditions of nearly constant pH, 0.1 per cent Aerosol OT increases the phenol coefficient of germicides in the F. D. A. method (special), *Staph. aureus*, 37° C., as follows: phenol, from 1.0 to 1.8; U. S. P. cresol, from 2.4 to 4.4; U. S. P. tincture of iodine (strong), doubtfully and irregularly from 3.7-4.3 to 4.3-5.5. The apparent phenol coefficient of phenylmercuric nitrate (not corrected for bacteriostasis) is increased from 166 to 1,300. The characteristics of Aerosol OT as a potentiating agent for germicides are described.

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MENINGOCOCCUS GROUPING

Note on Experience with the Capsular Swelling Test

Edith A. Beckler, B.S.*
Boston, Mass.

FOR approximately forty years attempts have been made to differentiate strains of meningococci by means of serologic reactions. The early work of Kutscher (1906),[1] Dopter (1909),[2] Elser and Huntoon (1909),[3] and Arkwright (1909),[4] made use of agglutination and absorption tests. Similar methods used by later workers, Gordon and Murray (1915)[5] and Nicolle, Debains, and Jouan (1918)[6] enabled the investigators to divide the meningococci from epidemic and sporadic cases into four groups. Griffith (1917)[7] and Scott (1918)[8] divided them by agglutination into two main groups. Branham, Taft, and Carlin (1931)[9] made detailed studies of meningococci isolated in the United States and indicated the difficulties in attempting a serologic classification when separation into four types by agglutinin absorption was done.

A method of differentiation described by Clapp, Phillips, and Stahl (1935),[10] based on the capsular swelling exhibited by certain strains of meningococci with homologous serum (rabbit), has been strangely disregarded by laboratory workers, although Cooper and Walter (1939),[11] of the New York City Health Department, apparently had some success with it. Einhorn (1937)[12] described the application of the Neufeld reaction to the identification of types of meningococci. Branham called attention to it in *Diagnostic Procedures and Reagents* (1941) and more recently (1945)[13] advised its use.

Because of the increase in the number of cases of meningococcus meningitis in Massachusetts during the past two years, it was possible for the Bacteriological Laboratory of the Massachusetts Department of Public Health to evaluate this method. From Jan. 1, 1943, to April 30, 1945, a total of 195 specimens of spinal fluid was examined for meningococci. They were found in seventy-three specimens, of which sixty-four were spinal fluid and nine were cultures on blood agar plates or some medium on which the sender had made cultures of the spinal fluid. The majority of these specimens were received by mail and were several hours old when delivered at the laboratory. Autolysis of the meningococci did not appear so extensive as to prevent the cultivation of the organism in most instances where it was believed to have been present.

The laboratory procedure was as follows: Upon the receipt of the specimen, a Gram stain was made of the fluid, for orientation. If any gram-negative diplococci were seen on examination with the oil immersion objective at a magnification of 900 to 1000, a capsular swelling test was set up at once with the spinal fluid. If no organisms resembling meningococci were found, the spinal fluid was centrifuged for an hour and then the sediment examined by Gram stain. Quite often other bacteria such as *Hemophilus influenzae*, pneumococci, or streptococci were found and appropriate tests were made for their identifica-

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*Chief of Laboratory, Massachusetts Department of Public Health.

tion. Even if no bacteria were seen in the sediment, a capsular swelling test for meningococci was set up, as a diplococcus overlooked in the stained preparation might thereby become apparent.

The capsular swelling test was made in a manner similar to that used for the Neufeld reaction in pneumococcus typing. A loopful of spinal fluid or sediment was placed on a plane slide and to it were added one loopful of the meningococcus Group 1 monovalent rabbit serum and a loopful of Loeffler's methylene blue. The mixture was stirred and a cover glass (⅞ inch square, No. 1) was placed over the wet preparation. Another mixture was made using Group 2A serum. The meningococcus sera used were obtained from the Lederle Laboratories. Cultures received on solid media were rubbed up in a drop of normal saline solution when the test was made. These preparations were examined with the oil immersion objective and the light partially dimmed. They were thoroughly examined when first made and if no capsular swelling was observed, they were re-examined after an hour. In a positive reaction the capsule around the blue-stained diplococcus was enlarged, was of a greenish-gray color or ground-glass appearance, with a thin dark line at the periphery. This reaction was seen with meningococci of Group 1 (types 1 and 3) and of Group 2A; up to the present time, no reaction has been observed with the Group 2 or Group 4 strains. If a reaction was seen with the direct preparations of the fluid or sediment, a report of Group 1 or Group 2A meningococcus was telephoned at once to the sender. The capsular swelling with the homologous antiserum (rabbit) seemed to be a specific reaction, for in every instance the cultural, agglutination, and repeated quellung tests confirmed the original diagnosis. If no reaction was seen in the direct preparations of the fluid or sediment, cultures were made from the sediment on chocolate agar, starch agar, Loeffler's blood serum, Avery broth, etc. in duplicate and incubated at 34° C. One set was incubated in the air and the other in an atmosphere containing about 10 per cent carbon dioxide, satisfactorily obtained in a "candle jar."

Occasionally mouse inoculation was used, especially when no organisms were found on microscopic examination. The technique employed was Miller's mucin-mouse method as described by Sulkin (1939).[14] This method may enable one to demonstrate capsular swelling within a few hours after the intraperitoneal inoculation of the mouse.

The cultures were examined for bacteria after from eighteen to twenty-four hours' incubation and thereafter every day for several days. Whenever any gram-negative diplococci resembling the meningococcus were seen in any culture or mouse peritoneal exudate, capsular swelling tests were made.

In this study, of the seventy-three specimens in which meningococci were found, fifty-four (74 per cent) were grouped directly without waiting for cultural growth; the remainder (26 per cent) grouped after incubation of the cultures, usually on the following day. There were sixty-five strains of meningococci belonging to Group 1 and five to Group 2A. Three specimens contained Group 2 meningococci which could be grouped only by agglutination tests.

Results which were doubtful were obtained (1) in the examination of specimens which were obviously contaminated when received, usually in non-sterile containers (because of overgrowth of spore-bearing bacilli or staphylococci, a few meningococci might have been obscured); (2) in the examination of specimens in which an occasional gram-negative diplococcus was seen in the

spinal fluid which could not be cultivated and showed no capsular swelling. However, if an occasional meningococcus is seen with a swollen capsule, that would appear to establish the identification without subsequent cultivation.

The simplicity of the capsular swelling test should make the grouping of meningococci, to which it is adaptable, a common practice in laboratories, so that the occasional need of the auxiliary use of homologous monovalent antimeningococcic serum can be met with as little delay as possible.

These examinations were made by the bacteriologists of the Bacteriological Laboratory of the Massachusetts Department of Public Health, to whom the writer is greatly indebted.

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THE DIAGNOSTIC SIGNIFICANCE OF INCLUSION BODIES IN RABIES AND CANINE DISTEMPER

MAJOR HAROLD RIFKIN, MEDICAL CORPS, LIEUTENANT COLONEL EMIL B. CEKADA, MEDICAL CORPS, LIEUTENANT MEYER ZARROW, SANITARY CORPS, ARMY OF THE UNITED STATES, LIEUTENANT COMMANDER DONALD G. HENDERSON, UNITED STATES NAVAL RESERVE, AND MAJOR JACK O. WHITEHEAD, VETERINARY CORPS, ARMY OF THE UNITED STATES

IN A LARGE island base in the South Pacific Area, rabies has never been known to occur. An analysis of communicable diseases existing on this island by the local health authorities reveals a complete absence of rabies. Due to the increased war shipping and the opening of this area as an important harbor, together with the introduction of dogs from the continental United States, the problem of rabies loomed on the epidemiologic horizon.

The incidence of canine distemper in this island base is extremely high. Native laboratory workers have informed us that the incidence is as high as 60 per cent among the native dogs. These dogs present the respiratory, gastrointestinal, and urinary findings so characteristic of the disease. We have, during the course of an investigation for the presence of rabies, found an encephalitis complicating canine distemper. This has produced nervous symptoms and signs almost identical with the clinical picture seen in rabies. Inclusion bodies, similar but not characteristic of the Negri bodies found in rabies, have been found in various portions of the brain. We present this paper to indicate the simulation of canine distemper to rabies and its importance in differential diagnosis.

Two dogs, who had been pets of a marine group situated on this island base, suddenly became restless, excitable, and vicious. They presented focal epileptic convulsions and had bitten six marine soldiers. No history could be elicited regarding respiratory and gastrointestinal findings. The marines bitten were given the Pasteur treatment. One dog was immediately shot by the Marine personnel and sent to a neighboring Naval hospital for autopsy examination. They amputated and buried the head of the second dog.

Protocol FH-A1.—At autopsy, the body externally revealed no diagnostic findings. The heart revealed slight congestion. No valvular lesions were visible. The lungs revealed a hemorrhagic consolidation, focal in distribution, throughout the left lower lobe. Raised, thickened gray-white nodules which, on close examination, consisted of thickened bronchiolar walls were present. A thick mucoid exudate could be expressed from the cut surfaces. The surrounding lung tissue was edematous, boggy, and congested. The remaining viscera were intact. The brain was not weighed. The meninges and the brain substance revealed congestion and moderate edema. Coronal sections throughout the brain revealed congestion of the small vessels, with petechial hemorrhages throughout. Tiny petechial hemorrhages, measuring 2 to 3 mm. in diameter, were distributed through the motor cortex, the mid-brain, pons, medulla, hippocampus, and cerebellum. Touch preparations from these areas were made.

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They were stained by Mann's and Seller's methods. Smears from the motor cortex, medulla, and cornu ammonis revealed oval bodies, measuring 2 to 10 μ in diameter, occurring singly or multiple (three to five) in the cytoplasm of the ganglion cells. The cytoplasm stained a pink-red with Mann's stain and revealed small clear vacuolated-like bodies in its interior. No characteristic central basophilic chromatin granulations were visible however. At this time, these were interpreted as Negri bodies, and a diagnosis of rabies was made. Hematoxylin-eosin stained sections from various portions of the brain revealed a moderate edema, with moderate congestion and hemorrhage. Slight perivascular mantling with small lymphocytes and polymorphonuclear leucocytes was found most prominent in the mid-brain. Tiny focal areas of nerve cell degeneration, characterized by swelling of cytoplasm and nucleus, in turn, surrounded by an early glial proliferation were present. Sections stained by Mann's stain revealed identical inclusion bodies. The picture did not fit the morphologic interpretation of rabies, but the inclusion bodies appeared strongly indicative of such a diagnosis. Animal inoculation was not performed at this time.

As a result of this preliminary investigation, the head of the second dog was disinterred and turned over to an Army general hospital laboratory for verification of diagnosis.

Protocol GH-A1.—Examination of the brain revealed moderate congestion and edema of the brain substance and overlying meninges. There was moderate autolysis throughout. Coronal sections throughout the brain revealed vascular congestion with petechial hemorrhages. Touch preparations, utilizing the method described, revealed similar inclusion bodies in the ganglion cells of the motor cortex, the temporal lobe, the hippocampus, pons, and mid-brain. They were also found in the Purkinje cells of the cerebellum. Histologic examination revealed an intense congestion with perivascular hemorrhages, in turn surrounded by focal areas of degeneration, characterized by nuclear pyknosis and swelling of the cell cytoplasm. In some areas, there were perivascular collars of lymphocytes and neutrophilic leucocytes. These were most prominent in the region of the mid-brain and medulla. Intracerebral inoculations of a saline suspension of brain tissue, including motor cortex, cornu ammonis, and cerebellum, were performed, utilizing a guinea pig, mouse, and rabbit, respectively. Leach's methods with slight modifications were employed. The animals were watched for a period of sixty days. They at no time presented clinical findings suggestive of rabies, and at the end of this period were alive and healthy.

The possibility that we were not dealing with true rabies but with an encephalitis, probably viral in origin and associated as part of the clinical syndrome of canine distemper, was now entertained. Fortunately, a member of the Veterinary Corps had brought to the laboratory four dogs which were clinically diagnosed as having distemper but which presented, in addition to the respiratory and gastrointestinal findings, symptoms suggestive of brain involvement. These dogs were watched. They died within a period of seventy-two hours. The autopsy findings in each of the cases were almost identical. We present one such representative case.

Protocol GH-A3.—The dog presented a mucopurulent discharge in both nostrils. The right and left pupil each measured 4 mm. in diameter. No other noteworthy findings were noted on external examination. The heart presented no remarkable features. The lungs were congested and edematous. There were irregular focal areas of deep blue-red consolidation measuring 3 to 4 cm. in

diameter and raised above the general surface for a varying distance. These were surrounded by blue-gray depressed areas of atelectasis. These were most prominent in the right and left lower lobes. The bronchiolar walls were thickened, and a thin mucopurulent exudate exuded from the cut surfaces. The larynx and trachea revealed an edematous mucous membrane. Scattered throughout were numerous irregularly distributed petechial hemorrhages. There was a thin mucopurulent exudate in the lumen. The bronchi and bronchioles were congested and edematous. The walls were thickened and indurated and contained a semiviscid gray-white exudate. The bladder presented numerous petechial hemorrhages throughout the mucosa. A number of these measured up to 1 cm. in diameter. There was no evidence of trabeculation or diverticula formation. The remaining viscera presented no prominent findings. There was congestion of the cerebral meninges. The brain substance itself was edematous and congested. Coronal sections throughout revealed a petechial hemorrhage, most prominent in the mid-brain and medulla, associated with focal areas of gray-yellow degeneration. Touch smears from various sections of the brain revealed identical inclusion bodies to those found in the previous cases. Touch smears from the trachea, bronchi, and mucosal wall of the bladder revealed similar inclusion bodies. Microscopic examination revealed findings in the brain similar to those described in the previous two protocols: namely, a moderate congestion, with perivascular collaring of lymphocytes and neutrophilic leucocytes, focally distributed petechial hemorrhages, and areas of nuclear pyknosis and cytoplasmic swelling surrounded by a zone of neuronophagia. Bacterial stains with the Brown-Brenn[1] method revealed no organisms. Microscopic examination of sections from the right and left lower lobes revealed extensive bronchiolar dilatation with desquamation and ulceration of the mucous membrane. The lumen was filled with a combined mononuclear and polynuclear cellular exudate. The bronchiolar and alveolar walls revealed a round cell infiltration. Small numbers of alveoli contained scattered monocytes and lymphocytes. Alveolar hyaline membranes were not visible. The alveolar septa were thickened and infiltrated with round cells. There was congestion of all the blood vessels, including the alveolar capillary bed. An occasional inclusion body was seen in the remnant epithelial lining of the small bronchioles.

Intracerebral inoculation of a guinea pig, rabbit, and white mouse, respectively, with sterile saline emulsions of involved brain tissue, produced no effect after sixty days.

The remaining three cases gave identical findings on macroscopic and microscopic examination and with animal inoculation.

COMMENT

The cases presented are of diagnostic and epidemiologic importance. It is apparent that we were not dealing with rabies but with canine distemper or a variant of this disease complicated by an encephalitis. Nervous symptoms due to encephalitis are apparently not new findings in canine distemper. Zinsser and Bayne-Jones[2] record this in their discussion of distemper of dogs. The finding of inclusion bodies in the ganglion cells of the brain in meningoencephalitis, secondary to canine distemper, has been recorded by Kelser.[3] It appears therefore that any inclusion bodies found in the brain are not directly diagnostic of rabies. Negri bodies have a distinct specific structure with the central basophilic staining chromatin granulation, a prominent finding. It is not meant to infer that prophylactic and therapeutic measures

should not be undertaken but rather that careful watching of the dog be performed; that search for the specificity of the inclusion bodies be made; that animal inoculation be performed in all instances for confirmation; and should the animals be brought to autopsy, scrapings of the trachea, bronchi, and urinary bladder be made in order to rule out canine distemper with secondary encephalitis.

SUMMARY AND CONCLUSIONS

1. Six dogs are described with nervous symptoms suggestive of rabies but with morphologic evidence of an encephalitis.

2. In all cases, inclusion bodies, similar but not characteristic of Negri bodies, were found in the ganglion cells of the cerebrum, mid-brain, pons, medulla, and in the Purkinje cells of the cerebellum.

3. In five dogs, there was evidence of a "virus" pneumonia. Similar inclusion bodies were found in tracheal, bronchial, and vesical smears in four of these cases.

4. Intracerebral inoculation of guinea pigs, mice, and rabbits, utilizing a sterile suspension of involved brain tissue from five of these cases, did not confirm a diagnosis of rabies.

5. Canine distemper with complicating encephalitis may simulate rabies clinically and morphologically.

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OCULAR DISTURBANCES IN RIBOFLAVIN DEFICIENCY

TOM D. SPIES, M.D., DANIEL J. PERRY, M.D., ROBERT C. COGSWELL, M.D., AND WALTER B. FROMMEYER, M.D.
BIRMINGHAM, ALA.

EARLY Italian writers on pellagra, notably Soler[1] in 1791, included, among other symptoms of the disease, inflammation of the cornea and corneal opacities. In the light of present knowledge, these lesions might be interpreted as manifestations of riboflavin deficiency.

That certain ocular conditions might be due to a deficiency of one of the B complex vitamins (B_2 or G) was suggested by observations on rats maintained on a diet low in this vitamin. Between 1928 and 1931 numerous investigators[2-4]

University of Cincinnati Studies in Nutrition at the Hillman Hospital, Birmingham, Alabama. From the Department of Internal Medicine, University of Cincinnati.

The tremendously heavy expenses of the general nutrition study have been borne by grants from a number of philanthropic persons, foundations, and commercial concerns, without which this study could not have been made. The necessary expense of frequent observations of the patients in their homes and in the clinic over a long period of time has been defrayed by grants from the Nutrition Foundation, by Merck and Co., Inc., and by the Lions Club of Alabama through the cooperation of the Alabama Sight Conservation Association.

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referred to ophthalmia and conjunctivitis observed in rats deficient in vitamin B_2 or G. In 1931 Day, Langston, and O'Brien[5] reported alopecia of the eyelids, lacrimation, conjunctivitis, thick, swollen, inflamed lids, cataract, and anterior interstitial keratitis in rats on a vitamin G-deficient diet. These findings were confirmed by a number of investigators. In 1937 Day, Darby, and Langston[6] reported that pure riboflavin, natural or synthetic, would prevent the keratitis, cataract, and other ocular manifestations of vitamin G deficiency. The following year the same laboratory reported that the parenteral administration of pure riboflavin to rats with ocular manifestations of riboflavin deficiency resulted in the rapid disappearance of the keratitis and in many instances the arrest of the cataractous changes. Day and Darby[7] in 1938 reported that severe keratitis was seen in all animals receiving a limited amount (30 gamma) of pure riboflavin weekly but that cataract was less common than in animals receiving a diet from which riboflavin was rigidly excluded. Early in 1939 Bessey and Wolbach[8] described their important observations on the cornea of rats whose diets were deficient in riboflavin. In their summary of a very careful histologic study of the vascularization of the cornea of the rat with riboflavin deficiency, Bessey and Wolbach stated: "Vascularization of the cornea of the rat in the absence of antecedent pathology is probably a specific and the most reliable criterion of riboflavin deficiency." A little later Eckhardt and Johnson[9] reported ocular changes in rats on a riboflavin-deficient diet. They stated, "Keratitis and vascularization of the cornea were more consistent ocular changes than cataract and were improved by the addition of riboflavin to the diet."

The first definite statement in regard to certain eye symptoms in persons with riboflavin deficiency seems to be that of Spies, Bean, and Ashe in May, 1939.[10] They stated that patients with riboflavin deficiency complained of visual disturbances which disappeared within forty-eight hours following the administration of riboflavin and that these symptoms returned within from ten to twenty days after riboflavin was discontinued. This statement was amplified in a paper published by Spies, Vilter, and Ashe in September, 1939,[11] in which attention was called to heretofore undescribed ocular manifestations of riboflavin deficiency in human beings. These manifestations included bulbar conjunctivitis, dilatation of the conjunctival vessels, burning of the eyes, lacrimation, failing vision, and extreme photophobia. These symptoms, in many persons, disappeared following riboflavin therapy. Soon many articles appeared describing various ocular manifestations associated with riboflavin deficiency. In November, 1939, Sydenstriker, Geeslin, Templeton, and Weaver[12] reported that conjunctivitis and photophobia in a patient with riboflavin deficiency improved following riboflavin therapy. In January, 1940, Kruse, Sydenstriker, Sebrell, and Cleckley[13] reported ocular lesions in nine patients with other symptoms of riboflavin deficiency and stated that the principal manifestation was keratitis. Following the administration of riboflavin, the corneal lesions improved or disappeared and reappeared on cessation of riboflavin therapy. In May, 1940, Johnson and Eckhardt[14] reported that in a series of thirty-six patients with rosacea keratitis, only four failed to respond satisfactorily to oral riboflavin therapy. In June, 1940, Sydenstriker and his associates[15] again reported on the visual changes associated with riboflavin deficiency. They stated that photophobia, dimness of vision, and impairment of visual acuity were relieved promptly by riboflavin therapy, in some instances before visible changes occurred in the cornea. Using the slit lamp in examination of the cornea, they observed that proliferation and engorgement of the limbic plexus was the earliest

lesion. This progressed to superficial vascularization of the cornea and the production of interstitial keratitis. They found that congestion of the sclera, vascularization, opacities of the cornea, and abnormal pigmentation of the iris responded rapidly to the administration of riboflavin.

Numerous reports have continued to appear in the medical literature, some describing various ocular manifestations associated with riboflavin deficiency and others denying their existence. Today there is considerable controversy on the subject, and a wide divergence of opinion exists among investigators in the field. Some physicians doubt that riboflavin deficiency affects the eyes, whereas we, as well as many other investigators, believe that it gives rise to ocular lesions and that a great many persons are affected. It is easy to see the differences in the point of view between the two groups. It is most difficult to overcome them, but success in doing so is vital to proper medical care.

It is hoped that this paper will aid the physician in the recognition and treatment of the ophthalmic lesions of riboflavin deficiency. It is concerned only with the study of ocular lesions in patients whom we believe to have riboflavin deficiency. This study, which was begun in 1938 and extended through 1944, is based entirely on naturally occurring lesions which responded to riboflavin therapy.

In 1938 and 1939 we had conferences with Dr. M. A. Blankenhorn, Professor of Medicine, and Dr. Derrick Vail, Professor of Ophthalmology, University of Cincinnati, in regard to using the slit lamp in examining patients in the Nutrition Clinic of the Hillman Hospital in Birmingham, Ala. As a result of these conferences, Dr. William Ashe and Dr. Barnet Sakler came to Birmingham for a short period of study, Dr. Ashe to aid in general medical aspects of the problem and Dr. Sakler to examine the cornea. Dr. Sakler noted the same symptoms we had observed and thought that the cornea should be studied in a considerable number of subjects to determine the relationship of riboflavin to vascularization of the cornea. He believed the problem required extensive and intensive collaboration of both ophthalmologists and physicians interested in nutrition; accordingly, Dr. Vail sent Dr. K. W. Ascher, of the Department of Ophthalmology of the University of Cincinnati, to our Nutrition Clinic at the Hillman Hospital in Birmingham in 1940 and 1941. A report by Dr. Vail and Dr. Ascher was published in the *Transactions of the American Ophthalmological Society,* Seventy-Eighth Annual Meeting, 1942.

The collaboration with the Department of Ophthalmology can be summarized by stating that the ophthalmologists were, by training, skillful in the use of the slit lamp and in the interpretation of the ocular findings, whereas our general knowledge of experimental medicine and nutritional deficiency diseases enabled us to point out general features which otherwise might have been disregarded. Out of this collaboration has come a much more logical development of the study than could have been achieved without it.

METHOD OF STUDY

In the Nutrition Clinic we examine several thousand persons each year. Many of these persons have no clinical evidence of dietary deficiency disease. If, from their dietary and medical history, we can discover no reason for their having a nutritional deficiency and if the physical examination reveals no evidence of deficiency, they are not registered as patients in the Nutrition Clinic but are referred elsewhere for diagnosis and treatment. In some instances they return to their own physicians and in other instances to other departments of the Hillman Hospital. Those who have no diagnostic evidence of dietary

deficiency at the time but whose histories and symptoms suggest that they might be developing dietary deficiency disease are observed frequently. These persons and those with clinical dietary deficiency diseases are carefully studied to obtain all possible information on their past and present nutritional status and health by means of thorough medical and dietary histories, repeated physical examinations, and indicated laboratory determinations. A detailed description of the methods we have found useful in obtaining such information was published recently.[16] The dietary histories revealed that 90 per cent of the patients who came to the Nutrition Clinic had eaten diets of varying degrees of inadequacy. In analyzing the data obtained from the medical histories and physical examinations, we found that 70 per cent of the patients having clinical deficiency disease complained of visual disturbances.[11] From this group in 1938 we began selecting patients for study of their ocular symptoms.

We felt that more comparable cases could be selected if the group of physicians selecting them was not too large and if there were frequent consultations among the physicians concerned. Accordingly, all the cases were selected by Dr. Richard W. Vilter, by Dr. William B. Bean, and by us. The criteria for the initial selection of patients were:

1. Ingestion of a diet inadequate in riboflavin
2. No apparent means of changing the diet significantly for a number of years
3. History of one or more of the following symptoms: burning and itching of the eyes, failure of visual acuity, photophobia, lacrimation, conjunctivitis, night blindness
4. Ocular lesions characterized by abnormal dilatation of conjunctival and corneal blood vessels and, in many instances, by corneal ulceration

Since our interest was concerned primarily with endemic riboflavin deficiency, we excluded most of the patients with known organic disease. We did include ten persons with hypertensive heart disease and twelve who had trauma to some part of the body other than the eyes. All persons with traumatic disease of the eyes were excluded, as were all persons who had syphilis, tuberculosis, chronic alcoholic addiction, diabetes mellitus, and any known disease of the alimentary tract. Many persons with ocular lesions also had clinical evidence of advanced beriberi, pellagra, or scurvy which required immediate and intensive treatment. These persons were likewise excluded from this special study as we wished to eliminate the possibility that the treatment given for one of these coexisting deficiency diseases might affect the natural course of the ocular lesions and their response to riboflavin therapy. We have observed that this can occur.

We freely admit that we do not know what specific ocular symptoms and lesions are caused by riboflavin deficiency. Nevertheless, that many such manifestations do respond to riboflavin therapy must be granted. It is only by determining the effect of riboflavin therapy on these ocular manifestations that a diagnosis of riboflavin deficiency can be made. As can be seen from the criteria used for selecting the patients for this particular study, we made every attempt to exclude persons whose eye involvement might be due to other causes. That this method of diagnosis and treatment is unsatisfactory we know, but until more reliable methods are available, we can recommend no better way of relieving and often rehabilitating persons in whom ocular disturbances of this special type have developed.

After excluding the above groups, we arbitrarily selected patients until we had 500. Two hundred with moderate eye involvement were given riboflavin

by mouth only. Three hundred with more severe eye involvement were given riboflavin at least once intravenously. It is with this group of 300 patients that this report is concerned.

Some of these patients were admitted to the hospital, but the majority remained at home and came to the clinic for observation and treatment. They were observed before, during, and after therapy. In many cases, slit-lamp examinations were made. Bacteriologic studies were made in fifty patients. These included smears and cultures taken from the lesions and from the exudate from the eyes. The blood level of riboflavin was determined in fifty patients. Urine examinations were done on the first 150; then it was found that the urinary excretion of riboflavin was so low that it could not be detected, and it did not seem worth while to study it in additional patients. A few of the early determinations of riboflavin in the urine were made by Dr. William Ashe, using the Emmerie method.[17] Most of the urine examinations were made by Dr. S. S. Sanders, who also made all the blood determinations, using the microbiologic technique of Snell, Strong, and Peterson.[18]

The patients in this study included males and females, both white and Negro, ranging in age from 8 to 92 years. Throughout the course of the study, we obtained a dietary history at frequent intervals and made repeated physical and eye examinations.

OBSERVATIONS

The dietary histories obtained at frequent intervals revealed that, while a few patients had eaten good diets until only a year prior to the onset of illness, most of them had subsisted on inadequate diets for many years. In most instances the income was low, and in order to have enough food to satisfy hunger, they had purchased low cost foods, such as cornmeal, flour, dried beans, sugar, and fat pork, foods that are relatively inexpensive. Higher cost foods, such as milk, meat, eggs, green vegetables, and fruit, either were never included or were included at infrequent intervals. In a comparatively small number of cases, loss of appetite or food idiosyncrasies were responsible for the patients' having had inadequate diets. Routine nutrition histories, valuable as they are in suggesting whether or not the diet has been adequate, do not indicate the degree of the deficiency. Accordingly, in 100 of the 300 patients, we made repeated detailed studies of the diet. Observation of these patients over a long period of time convinced us that we could rely on the information obtained concerning their diets. Patients were instructed to keep a daily record of their food intake for a week at different seasons of the year. During these times, frequent visits were made to their homes to check their food supply and to determine how their food was prepared and served. From these data, the average daily intake of nutrients was calculated. The results of this study are shown in Fig. 1. Note that the diets of these patients were generally deficient and that they supplied only 36 per cent of the recommended allowance of riboflavin.

The most conspicuous and consistent early complaint elicited from analysis of the medical histories was that of weakness and fatigability. Frequently these patients were unable to participate in any activity and most of them had been unable to work regularly for years. A few of them had been able to work for a few hours and then were exhausted for a number of days. Very common complaints were nervousness, insomnia, headache, burning and aching of the legs and feet, burning and cramping of the stomach, constipation, burning of the skin over various parts of the body, itching and burning of the eyes, and failing

vision. As the seasons and years passed, these symptoms waxed and waned in intensity, but in almost every patient there was a wide variety of symptoms which ranged from numerous ill-defined complaints to the final development of a diagnostic lesion.

The observations in this paper are concerned primarily with the eyes. It is of considerable interest, however, that 154 of these patients had cheilosis sometime during the years they were observed, fifteen of them on only one side of the mouth. The degree of cheilosis ranged from paleness at the angles of the mouth to maceration and piling up of grayish-white tissue. When this tissue was scraped off, it left a pink background. In some patients these lesions, in the later stages, were covered with dry yellow crusts which could be removed without causing bleeding. None of the patients in this group had the abnormal redness of the lips, particularly along the line of closure, which is sometimes seen in riboflavin deficiency. In two instances, the tongue was magenta in color. In twelve patients, there was a fine, scaly, somewhat greasy desquamation around the nasolabial folds.

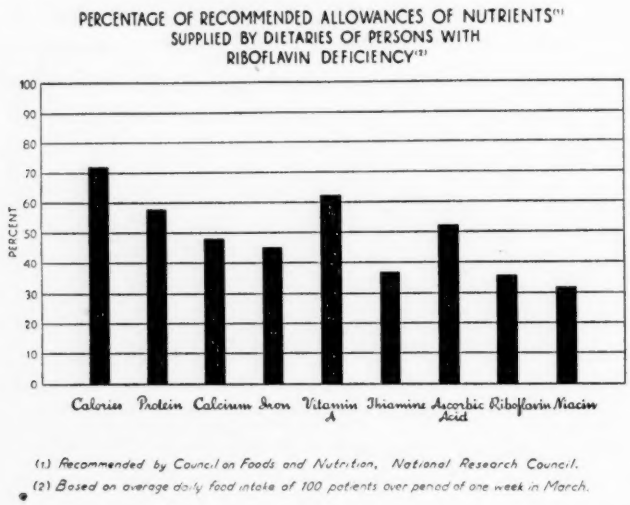

Fig. 1.

For the most part, when these lesions occurred, they paralleled the ocular lesions in severity. They will not be described further in this paper. Suffice it to say that the ocular manifestations frequently occurred with no evidence of cheilosis and vice versa. The visual symptoms in practically all the patients were heralded by a feeling of dryness in the eyes, which was followed by burning and itching, sometimes by photophobia and lacrimation. A frequent statement volunteered by the patient was that the eyes felt "full of sand." Every patient had objective ocular disease with involvement of the conjunctivae, cornea, and sometimes of the iris. Those who did not have these lesions were not included in this study. Conjunctivitis sometimes was the sole manifestation and was shown by increased visibility of the vessels of both the bulbar and palpebral conjunctivae, apparently due to congestion and dilatation. This gave the eye a reddened appearance. The redness was more apparent usually in the exposed or equatorial regions of the conjunctivae. In every one of the 300 patients who received parenteral riboflavin therapy, it was observed that small vessels encroached on the cornea at the scleral corneal junction. It is of passing interest to note that the circumcorneal injection was grossly visible in 80 per

cent of the patients, but this could be noted most readily with an ophthalmoscope or a hand lens. Interstitial keratitis was observed in 60 per cent of the patients and corneal ulceration in at least one eye in 53 per cent. The conjunctivae of most patients were thickened, particularly in the equatorial regions. Pterygia were especially common in this group. They became apparent in the eyes of some of the patients during the course of the study. The high incidence (almost 50 per cent) would suggest some kind of underlying association which might in some way be related to deficiency states.

It is to be admitted freely that the picture described is not one which is pathognomonic of riboflavin deficiency or one that can be immediately and specifically differentiated from superficial inflammations of the eye from numerous other causes. It is also very likely that experienced ophthalmologists seeing this number of patients might arrive at one or more specific diagnostic items which would strictly define the disorder. We are unable to do so. We have, however, made an effort to eliminate other etiologic disorders, such as the familiar vernal conjunctivitis, foreign bodies in the cornea, xerophthalmia, and such diseases of the uveal tract as iritis due to syphilis, tuberculosis, and rheumatic fever. As has already been stated, persons with evidence of serious complicating organic disease found by careful physical examination have not been included in our series.

Up to this time we find that the response to riboflavin is the most important diagnostic measure in the determination of the etiology of the ocular disease. This therapeutic test may in itself not be thoroughly valid because we have gained an impression that most inflammatory disorders of the superficial portions of the eye may be benefited to some degree by an excess supply of riboflavin to the organism. This impression may have been highly colored by the fact that most of the patients with whom we have been dealing have come from an area where nutritional disorders in general are endemic.

The riboflavin content of the blood was assayed by means of the microbiologic method of Snell, Strong, and Peterson,[18] using *Lactobacillus casei* and samples of whole blood. More recent reports[19, 20] indicate that this method is not specific in that there are apparently other substances in blood which stimulate the test organism in the presence of riboflavin. Our results, however, are reported as comparative rather than absolute values. We found that the blood of these patients with ocular disturbances ranged from 0.28 to 0.47 and averaged 0.43 gamma of riboflavin per cubic centimeter of whole blood. In a group of normal subjects the riboflavin content of the blood ranged between 0.29 and 0.56, and averaged 0.46 gamma per cubic centimeter of whole blood. Thus, there was not a statistically significant difference between the two groups. Within a few minutes after the injection of riboflavin, the concentration of riboflavin in the blood increased promptly as did the excretion in the urine. The riboflavin content of the urine was increased sufficiently to be visible even with the naked eye.

Smears and cultures were made from the exudate of the affected eyes in fifty consecutive cases. In forty patients we identified hemolytic staphylococci, streptococci, or xerosis bacilli. In nearly all instances the infection was a mixed one, and we found all these organisms in great abundance.

The management of the cases and their response to treatment are illustrated in the following three case reports.

CASE 1.—G. F., a 52-year-old Negro man, was brought to the Nutrition Clinic in July, 1942, complaining of severe pain in his eyes and inability to see.

Family history and past history were irrelevant.

Present illness: He had eaten an unbalanced, high carbohydrate diet most of his life. At the age of 2 years he was weaned and began eating the foods used by the rest of the family—corn bread, biscuit, sweet potatoes, grits, fat pork, and syrup. As he grew older he began eating pork chops and turnip greens two or three times a week. He never ate any other kind of meat or vegetable and never drank milk. He began working in a steel plant at 18 years of age. Despite his inadequate diet, his health remained "good" until ten years prior to the time he came to the clinic. At that time he was working hard at the steel plant during the day and taking care of his crippled mother at night. He was getting little sleep, lost his appetite, and his nerves "went down." He began to feel "scared" all the time and felt a sensation of impending calamity, although he did not know what it might be. He stated that the slightest noise, such as the dropping of a small rock or a piece of coal, made him "shake inside." He noticed no ocular symptoms at this time. His insomnia became worse and worse, and his employer became so alarmed about his condition that he had him admitted to a hospital where "they stuffed me with food." During the two weeks he was in the hospital, he gained ten pounds in weight and a great deal of strength; his appetite returned, and his insomnia and nervousness disappeared. The day after he was discharged from the hospital he went back to work. He remained well for two years; then he injured his foot and was unable to continue his work in the steel plant. He obtained light work on a farm in return for which he was given a house, rent free, and one dollar a week. He did not have enough money to buy meat of any kind and seldom had green vegetables, so his diet consisted almost entirely of corn bread, biscuit, sweet potatoes, fat pork, and syrup. Two months before he came to the clinic, he began having burning and "gas" in his stomach, so he restricted his diet to a pint of buttermilk daily, which was given to him by a neighbor, and three pieces of corn bread. About this time he felt a "scum" over his eyes, and he stated that they "felt full of trash." Soon they began "watering" almost constantly, and he could not go outside because the sunlight caused them to "shoot with pain." His eyes rapidly grew worse, and for two weeks prior to coming to the clinic he had been unable to work and had difficulty finding his way around the house.

Physical examination showed a thin, moderately well-developed Negro man who was extremely nervous, apprehensive, and undernourished. The subcutaneous tissues contained little fat. The skin over his entire body was dry and rough. His hair was very dry. He had no diagnostic lesions of pellagra but did have mild peripheral neuritis suggestive of beriberi. He cried a great deal and constantly held his hands over his eyes and would not remove them until we assured him that all the lights had been turned out. Extreme photophobia caused excruciating pain. He gradually allowed us to use a flashlight to examine his eyes and later a slit lamp and ophthalmoscope. The injection of the conjunctivae of both eyes was so pronounced that they resembled raw red meat. There were destructive lesions in the corneas of both eyes—in the right eye an ulcer about 4 mm. in diameter and in the left eye an ulcer about 2 mm. in diameter. Lacrimation was profuse during the entire examination. Purulent material on the lids and in the corners of the eyes adhered despite constant lacrimation.

He was given 15 mg. of riboflavin intravenously. Within twenty-four hours definite subjective improvement occurred. Within forty-eight hours lacrimation and photophobia had almost disappeared and there was a definite decrease in the size of the ulcers. The injections of riboflavin were continued for twelve days, and by the end of that time the ulcers had healed, leaving corneal scars. He felt much stronger and could see well enough to do a little work on the farm. He was advised to include in his diet daily at least one quart of milk, a serving of lean meat, two eggs, and two large servings of green vegetables and to come to the clinic once a week for observation. For the next two years he was unable to get the foods he should have and continued to eat an inadequate diet. During this time he had eleven recurrences of lacrimation, photophobia, and injection of the conjunctivae. Each time he was given 15 mg. of riboflavin intravenously. With each recurrence the symptoms appeared in the same sequence, but the deficiency was never allowed to progress until ulceration recurred. Since the last recurrence his salary has been increased and he has improved his diet. He has had no recurrence of symptoms for six months. At the present time he is working every day, and when we last saw him, shortly before the publication of this paper, his eyes were in good condition except for corneal scars.

This case is illustrative of a man who had a poor diet, worked long hours, had insomnia, and who gradually developed the initial nervous syndrome so often relieved with thiamine and the photophobia and corneal ulceration arising

from the deficiency of riboflavin. Following the administration of riboflavin, his eyes improved greatly, the photophobia disappeared, and the corneal ulcers healed. He continued to eat an inadequate diet and had eleven recurrences of the symptoms. At the time of each recurrence he was treated with riboflavin, and each time the symptoms disappeared. The response following the injection of riboflavin was so definite in every instance that he, like many others in this series, is an excellent example of the benefit which follows the administration of riboflavin to the properly selected patient.

CASE 2.—L. U., a 33-year-old albino negress, was brought to the Nutrition Clinic in August, 1943, because of intense pain in her eyes and inability to see. She was admitted to the Hillman Hospital for diagnosis and treatment.

Family history and past history were irrelevant.

Present illness: She had eaten a liberal, well-balanced diet all her life and had almost perfect health until she was 31 years of age. At this time she had an "abscess of the womb." During this illness she lost her appetite to the extent that she had a distaste for milk, meat, eggs, and vegetables, foods she had previously enjoyed. The day after her discharge from the hospital, she went to work as a cook in a restaurant. Her appetite remained poor, and instead of eating regular meals, she ate only occasional "snacks," such as cake, pie, sandwiches, or fruit. She became extremely nervous and began having pains all over her body, particularly in her stomach and head. She said she "flew off the handle" for no reason, whereas previously it had been unusually easy for her to get along well with people. At times she thought she was "going crazy." She worried constantly about her health and could not sleep. About a year after her operation, she noticed that light caused her eyes to pain and observed for the first time that they itched and "watered." These symptoms increased in severity and within two months from the time of their onset she could not look at a light. By this time her nerves were "all shot," and she screamed without provocation. She was unable to continue working and stayed at home where she spent day and night stretched out across her bed with her eyes closed, trying to find relief. She consulted a physician who prescribed an eye wash which she used for a month without relief; then she consulted another physician who also gave her an eye wash which she used in vain. By this time she was too weak to go to the bathroom and became a problem to the people with whom she lived. They brought her to the clinic and asked that she be admitted to the hospital since she was too weak to walk and was unable to see even to feed herself.

Physical examination showed an underweight Negro woman in excruciating pain. Inflammation of the conjunctivae of both eyes was obvious at considerable distance (Fig. 2). Her eyelids were matted with pus. Bacteriologic examination showed hemolytic staphylococcus and streptococcus and xerosis bacilli. On the right eye there was a corneal ulcer 4 by 3 mm. A consultant from the Ophthalmology Department believed that a staphyloma of the right cornea should be removed. This was done under local anesthesia.

She was given 10 mg. of riboflavin intravenously daily for ten days. Within twelve hours her symptoms were relieved considerably and there was a slight but definite decrease in the caliber of the conjunctival vessels. Her symptomatic improvement was much more definite than the objective changes, although the corneal ulcer had decreased in size. The profuse lacrimation disappeared, and on the tenth day after treatment was initiated, her eyes were no longer sensitive to light. The dramatic improvement following therapy is illustrated in the second picture taken twenty-one days after therapy was initiated (Fig. 3). Seven weeks later she returned to work as a cook in a restaurant. She was advised that she should include in her diet daily at least one quart of milk, a serving of lean meat, two eggs, and two large servings of green vegetables. Her appetite remained poor, however, and she continued to eat the same foods as she had prior to treatment. Physical examination two weeks after she returned to work showed bleeding hemorrhoids and fecal impaction, a condition that had not been present at the time she was examined in the hospital. She believed this to be a factor in her loss of appetite and her general nervousness. Although she has not altered her diet, she has been faithful about coming to the clinic for observation and treatment. In the past year she has had six recurrences of lacrimation, photophobia, and vascular dilatation of the conjunctival and corneal vessels. Each time she was given 10 mg. of riboflavin intravenously for ten days and each time her symptoms were relieved. Despite these mild but frequent recurrences, she has been able to work regularly.

Recently she has had more bleeding from the hemorrhoids and she has been referred to the surgical clinic. Blood studies made by Mrs. Calvin Koch and Mrs. Hayes Caldwell show that within the last five months the patient's red blood cell count has changed from 4.1 to 3.6 million per cubic millimeter and the hemoglobin from 8.8 to 8.2 Gm. per 100 c.c.

This case has been included for several reasons. In the first place, it is the only one in which it was necessary to do a local operative procedure on the eye. In the second place, this woman is one of four patients who, as far as we know, had no organic disease at the time she was selected for study but who later developed one. In the third place, she has an anemia which must be treated with iron, and we must determine whether or not she has liver disease or just what condition is causing the bleeding hemorrhoids. This case is so much like some we have seen in the General Hospital in Cincinnati where organic disease plays a more prominent role than it does among patients in the Nutrition Clinic in Birmingham that it seemed wise to include it.

We regard the data presented merely as a progress report. Despite the fact that by giving riboflavin we have been able to stop the severe ulcerative process in the cornea and have been able to rehabilitate the patient to the place where she can work regularly as a cook in a restaurant, we will not consider her completely rehabilitated until the hemorrhoids and anemia are successfully treated and until she improves her diet so that it is no longer necessary to give her riboflavin to prevent frequent recurrences of her ocular disturbances.

CASE 3.—L. N., a 68-year-old Negro woman, was brought to the Nutrition Clinic in February, 1941, complaining of excruciating pain in her eyes. She was unable to see well enough to walk unaided. She was admitted to the Hillman Hospital for diagnosis and treatment.

Family history and past history were irrelevant.

Present illness: She was in good health until six years prior to coming to the clinic. At this time she began having dizziness and shortness of breath. She consulted a physician who advised her to stop working. Until this time she had always had a good appetite and her diet had been liberal and varied, although it was high in carbohydrate. After she gave up her work as a laundress her only income was from a daughter who sent her small sums of money from time to time. It never averaged more than eight or nine dollars a month. With the money she had left, after paying for fuel and rent, she could buy little but corn meal, flour, syrup, dried beans, sweet potatoes, and fat pork, so she ate large amounts of these foods. She never had lean meat or milk and seldom had eggs or green vegetables. For six years her diet had been inadequate in all nutrients except calories (Fig. 4). Two years prior to the time she came to the clinic her eyes began to burn and "water" and she noticed that her vision was becoming "dim." Within a year she could not see to read. She kept the window shades down all day because the light hurt her eyes. For four months before she came to the clinic the pain in her eyes was so intense that she cried most of the time. She was unable to take care of her house or herself, so her granddaughter came to keep house for her.

When we first saw her, she held a cloth over her eyes which, she explained, kept the light out of her eyes. "My eyes feel like they is burned out with red hot pokers," she cried over and over again. It took a long time for us to gain her cooperation enough to allow us to examine her eyes. Exuding from both eyes was a mucopurulent discharge from which smears and cultures showed hemolytic staphylococcus and streptococcus and xerosis bacilli. The conjunctivae of both eyes were fiery red, and there was pronounced edema of both bulbar conjunctivae. Severe destruction of the corneas of both eyes, more severe on the left than on the right, was striking (Fig. 5). The lesions were so advanced that some observers thought she had "pyophthalmitis" and not a vitamin deficiency.

A complete physical examination showed that she was obese and had hypertensive heart disease. Her blood pressure was 180/110. The left cardiac border extended to the mid-axillary line.

Soon after the physical examination was finished she was given 15 mg. of riboflavin intravenously. Sixteen hours later she volunteered that her eyes were much less painful, and

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Fig. 2.—Note inflammation of conjunctivae of both eyes.

Fig. 3.—Note dramatic improvement twenty-one days after riboflavin therapy was initiated.

Fig. 5.—Note destruction of cornea, more severe in the left eye than in the right.

Fig. 6.—Note improvement forty days after initial riboflavin therapy.

there was a definite decrease in the redness of the conjunctivae. Slit-lamp examination showed that the vessels had decreased in caliber. She was given 15 mg. of riboflavin intravenously daily for two days. By the end of this time much of the redness of the conjunctivae had faded, and there was still further decrease in the caliber of the vessels and in the size of the ulcers in both corneas. She stated that she had scarcely any pain in her eyes. Dr. K. Ascher, who saw the patient at this time, agreed in general with our observations and noted contraction of the vessels in the new scar tissue. She was discharged after ten days in the hospital.

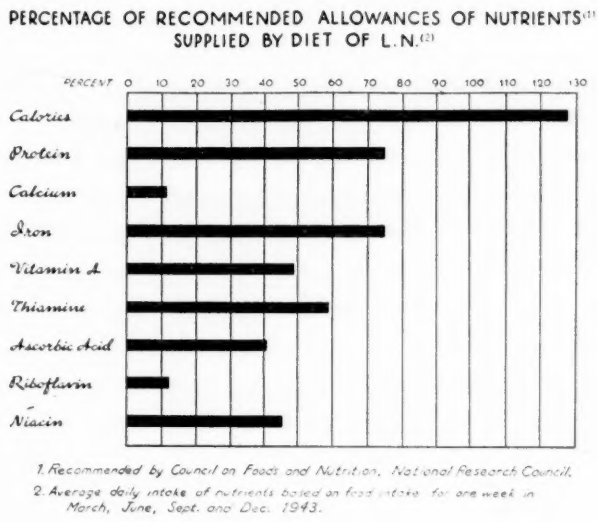
PERCENTAGE OF RECOMMENDED ALLOWANCES OF NUTRIENTS(1) SUPPLIED BY DIET OF L.N.(2)
PERCENT 0 10 20 30 40 50 60 70 80 90 100 110 120 130
Calories
Protein
Calcium
Iron
Vitamin A
Thiamine
Ascorbic Acid
Riboflavin
Niacin
1. Recommended by Council on Foods and Nutrition, National Research Council.
2. Average daily intake of nutrients based on food intake for one week in March, June, Sept. and Dec. 1943.

Fig. 4.

For the next two months her granddaughter brought her to the clinic weekly for observation (see Fig. 6, forty days after initial riboflavin therapy). At the end of this time all the redness of her eyes had disappeared and she could see well enough to come to the clinic alone. She went to visit her daughter in another town and did not come to the clinic again for six months. Her daughter was unable to provide her with a better diet than she had had at home. For five months she had no recurrence of the eye symptoms; then she developed photophobia and lacrimation which grew steadily worse. A month after the onset of these symptoms she returned to the clinic seeking treatment.

Examination of her eyes showed that the vessels of the conjunctivae and corneas were again dilated, and there was beginning ulceration in the corneas of both eyes. There was, however, much less inflammation and purulent exudate than when we had first seen her. Bacteriologic examination showed the same organisms as before. The administration of 15 mg. of riboflavin intravenously was again followed by dramatic improvement. This therapy was continued for ten days, and at the end of this time she could see well enough to read.

Although she was advised to include in her diet daily one quart of milk, a serving of lean meat, two eggs, and two large servings of green vegetables, she was unable to buy these foods and continued to eat a diet grossly inadequate in riboflavin. For the following two years she came to the clinic frequently for observation and treatment. During this time she had six recurrences of the ocular symptoms. Each time the symptoms recurred in the same sequence. The deficiency was never allowed to progress to the place where ulceration recurred. At the time of the first five recurrences she was given 15 mg. of riboflavin intravenously. The sixth time, 15 mg. was administered orally. Eight months ago she began getting an old-age pension and since then her diet has been adequate and she has had no recurrence of symptoms.

This patient's general health and strength are greatly improved despite the fact that she has organic heart disease. At the present time her blood pressure is 168/94. The corneas of both eyes are badly scarred and her vision is not perfect. Nevertheless, she can see well enough to read, to do her own housework, and to travel around town alone, whereas before treatment she had had to be led to the clinic.

This case is illustrative of a patient who had eaten a liberal and varied but especially high carbohydrate diet until she was 62 years of age, when illness

forced her to give up her job and she could no longer buy protective foods. As a result, her diet became not only unbalanced, but also grossly deficient in riboflavin. After eating such a diet for six years, she developed photophobia and lacrimation, severe conjunctivitis, and corneal ulceration of both eyes. Her eyes improved after intravenous riboflavin therapy, but large areas of scar tissue remained. She was unable to obtain a job and therefore could not buy proper food. In two years she had six recurrences of the ocular symptoms. Each recurrence was successfully treated with riboflavin before further irreparable damage to her eyes could develop. Then an old-age pension became available, which has made it possible for her to improve her diet, and she has had no recurrence of symptoms.

Included in this study were three cases of strabismus in which the part of the eye most exposed to light was the part most severely affected, showing that direct light had an effect. The response to treatment, however, was just as good as it was in the other patients.

DISCUSSION

During the past six years numerous investigators have described cheilosis or pérlèche and certain ocular manifestations as deficiency disease syndromes and have attributed them to deficiency of riboflavin in the diet. It is unfortunate that some physicians have regarded these manifestations as pathognomonic of riboflavin deficiency. As these lesions often disappear without any known specific changes having been made, it is difficult to assess the value of any substance in relieving them. Rest in bed or relief from insomnia often are followed by a decrease in the size or the disappearance of such lesions. When other diseases are present, the remedies directed toward treating these diseases often improve the general condition of the patient and the cheilotic lesions and ocular symptoms decrease or disappear. When pellagra is severe enough, the general nutritive state of the patient is often greatly impaired and cheilosis and ocular symptoms are frequently present. It is not uncommon to see general improvement in the nutritional status of the patient with pellagra and a disappearance of cheilosis and ocular lesions following only niacin therapy. Unless the diet becomes adequate, however, these lesions tend to return eventually, even when the patient is taking niacin. In this clinic we have seen cheilosis and ocular lesions identical in appearance to those described in this paper which did not disappear even after the patients had received from 2 to 10 Gm. of riboflavin over varying periods of time. These observations have led us to develop the working hypothesis that cheilosis is a clinical term denoting a lesion which may or may not be relieved by riboflavin therapy. It may be relieved by a number of agents that tend to increase the general health of the patient. In some instances it can be relieved by riboflavin alone. In regard to the ocular lesions we take the same point of view. To us it seems that there are certain fundamental anatomical structures involved in each case and that a number of different factors can predispose toward, or produce, such lesions.

In recent years much healthy skepticism has arisen in regard to the relationship of riboflavin deficiency to ocular lesions and cheilosis. This skepticism is chiefly due to the increasing number of papers in the medical literature in which it is reported that healthy subjects given a diet deficient in riboflavin for varying periods of time failed to develop such lesions. It has not been sufficiently stressed that riboflavin deficiency, if it exists as such, probably is the result of very long-continued subsistence on a diet low in riboflavin. Such diets

are common among persons living in some of the southern areas of the United States. This may explain why we see so many persons in our clinic who have cheilosis and ocular lesions which respond to riboflavin therapy. It also is conceivable that, with what has been called riboflavin deficiency, there may be associated, but as yet unidentified, lesions and that the cheilosis and ocular manifestations are only a part of the syndrome.

In many persons we have seen cheilosis without ocular involvement or ocular involvement without cheilosis. In many others we have seen involvement of only one eye or only one angle of the mouth. The degree of storage of riboflavin and other nutrients in the tissues of the body may play a prominent role in the appearance of these lesions. Year after year we hear these undernourished persons complain periodically of weakness, tiredness, irritability, and other vague symptoms; and in trying to arrange these symptoms into some sort of pattern, one is forced to the conclusion that the body can mobilize vitamins and transfer them from one tissue to another. All our studies on the riboflavin content of the blood tend to indicate that there is little or no fall in the concentration of riboflavin even in the presence of severe lesions. Nevertheless, that there is a tendency for the body to hold riboflavin is indicated by the fact that when we have given persons a diet deficient in riboflavin, the amount excreted in the urine decreases very rapidly and soon falls to normal.

The amount of riboflavin which is produced by intestinal bacterial synthesis may be very great. Naturally the type of bacterial flora concerned and the type of diet is very important. Although we have tried to discover whether or not the body can take riboflavin from the viable bacteria, we have little significant evidence either for or against such an hypothesis. Certainly, the flora of the intestinal tract changes in the later stages of deficiency diseases. It may well be that the intestinal tract, or the flora of the intestinal tract, is altered by deficiency disease so that persons with these diseases may absorb less riboflavin than do persons in good health. We must admit that, at this time, riboflavin deficiency is little understood. Nevertheless, the patients in this study had been ingesting a diet inadequate in riboflavin and they developed lesions which responded to riboflavin therapy. Irrespective of the mechanism, physiology, bacteriology, or biochemistry concerned, it is important for the practicing physician to know that such symptoms may arise and in many instances respond to the administration of riboflavin. At the present time we can do little more than recommend that the nutritional status of all patients with ocular lesions or cheilosis be carefully studied and that riboflavin be administered as a therapeutic test.

SUMMARY AND CONCLUSIONS

Three hundred patients who developed ocular disease after subsisting on diets deficient in riboflavin were selected and treated for brief periods of time with intravenous injections of riboflavin. There was a prompt elevation in the concentration of riboflavin in the blood and urine. This elevation was rarely sustained for more than a few hours. Within forty-eight hours there was some subjective improvement in all patients, perhaps because they were carefully selected in order to be certain that they had photophobia and were eating a diet low in riboflavin. The degree of improvement volunteered in 80 per cent of the cases was truly remarkable. Within this period there was observed a diminution in the caliber of the dilated vessels in the eye and a striking decrease in the photophobia and corneal ulceration. Accompanying this improve-

ment was a decrease in the number of hemolytic staphylococci, streptococci, and xerosis bacilli in the exudate from the eyes. Despite the fact that many of the patients included in this study have irreparable eye damage, they are relieved of pain and their vision is improved. Seventy-two per cent of these patients have been able to return to work after months or years of idleness. It is possible, of course, that some of these patients may have severe recurrences and lose their positions. Others may improve slowly and obtain work. We have not followed this group of patients long enough to be certain. These should be regarded only as tentative figures. In fact, we are hesitant about including them at all.

Most of the 300 patients continued to eat their usual inadequate diets with the result that 251 have had recurrences, the first recurrence appearing within from one week to two years after the initial period of therapy. The number of recurrences varied a great deal; a few of the patients have had only one recurrence, and one has had seventeen over a period of two years. In forty-nine patients there has been no evidence of clinical recurrence as yet, despite the fact that their diets have not improved. In all recurrences the symptoms tended to evolve in sequential order in the same patient, although the variety and severity of symptoms and the order of their appearance varied widely from patient to patient.

The observations reported in this paper and similar observations on other persons with ocular manifestations of riboflavin deficiency who have been treated with riboflavin administered either orally or intravenously have suggested four important concepts to the authors: The first is that the ocular manifestations of riboflavin deficiency are common in areas in which deficiency diseases are endemic; that they may occur in either sex and probably at any age (the ages of the patients in the series reported in this study varied from 8 to 92 years); that they may be unilateral or bilateral and may occur with or without cheilosis; and that they may or may not be associated with clinical evidence of other deficiency syndromes. The second is that the physician must administer riboflavin either orally or intravenously, not only through the most interesting part of the disease, namely, during the acute manifestations, but that he must continue this therapy until he is certain that the factors predisposing to riboflavin deficiency have been eliminated. The third is that the patient is likely to have recurrences of the ocular symptoms unless the physician instructs him to eat a diet that includes liberal amounts of lean meat, milk, green vegetables, and other protective foods and unless the patient not only understands but also follows these instructions. The fourth is that the physician who does not have a clear picture of the predisposing and precipitating factors in every case will find it more difficult to treat the patient successfully than the physician who has determined these factors and, insofar as possible, eliminated them.

Until more precise methods are available for assessing the degree of riboflavin deficiency in each patient, there is bound to be some controversy and confusion. Meanwhile it is our hope that these therapeutic concepts may be of some practical value.

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THE EFFECT OF BARIUM CHLORIDE ON THE HEART

A. M. Wedd, M.D., and H. A. Blair, Ph.D.
Rochester, N. Y.

IN THE course of investigating the action of certain drugs on various fundamental properties of cardiac muscle, observations have been made on the influence of barium chloride on the turtle heart. Part of the present interest in this drug was aroused by statements in textbooks of pharmacology that barium has a digitalis-like action (Sollmann, Goodman, and Gilman). In an earlier paper[3] in which a study of the action of digitalis was reported, the methods herein used were described in detail, and it was shown that the most constant action of digitalis is to shorten the refractory period of heart muscle. There has been quite general agreement that barium does increase the rate of beating, especially when rhythmicity is low, an action opposite to that of digitalis. This property led to the clinical use of barium for patients suffering from a slow idioventricular rhythm.

The effect on rate, contractility, and tonus was observed with whole auricles and ventricular strips suspended in a bath of Ringer's solution and the beat was recorded on a smoked drum. Observations were made on six auricles and

From the Department of Physiology, School of Medicine and Dentistry, University of Rochester.

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seven ventricles. In most instances the same tissue was tested on two or three successive days. A concentration of barium chloride 1:100,000 did not influence rate, and in some experiments 1:50,000 produced little or no change, but usually concentrations of 1:50,000 and 1:33,000 were effective. For the auricle the effect on rate appeared to depend on the initial rate of beating. In three experiments slight slowing occurred with the first trial, but when the same tissue was observed on the following day and rhythmicity was lower, the same concentration caused an increase in rate; one of these preparations which had an initial rate of 36 per minute was slowed to 32 by a concentration of 1:50,000; twenty-four hours later when the rate of beating was 26, it was raised to 38 by the same amount of the drug.

The effect on the rate of spontaneously beating strips of ventricle was similar to that for the whole auricle, but in general it may be said that the higher the initial rhythmicity, the less the barium effect, and in consequence the degree of rate change was usually greater in the ventricular strips than in the auricle. In one ventricular strip a concentration of 1:50,000 produced no change in rate; 1:33,000 raised the rate from 12 to 18 per minute, and 1:25,000 caused a further increase to 24 beats per minute. In only one of the seven ventricles did slowing occur, when the rate fell from 21 to 18 per minute with a concentration of 1:33,000; the next day the same concentration raised the rate from 15 to 34. However, the more marked action of barium seen in the older tissue was not always to be related to rate alone; in one experiment the fresh strip beat at 16 per minute and the rate was increased to 22 by 1:50,000 barium chloride; twenty-four hours later the same concentration raised the rate from 16 to 28. No satisfactory explanation can be offered for the more pronounced action of barium that was seen on the second and third days. The bath was always changed once or twice after each experiment, but no attempt was made to wash the tissue free from barium chloride. Moreover, as will be pointed out later, no similar behavior was observed for contractility.

When auricular tonus waves were initially present, they persisted after the introduction of barium into the bath, and at times that phenomenon was heightened. In one auricular preparation, tonus waves first appeared following barium, and that event was again observed on the second day. Tonus, indicated by diastolic length, often increased both in auricular and ventricular preparations; relaxation due to the drug was never seen.

The power of barium to produce extrasystoles and abnormal rhythms in the mammalian heart, which has been stressed by Rothberger and Winterberg[4] and Smith, Winkler, and Hoff[5] and which was found disturbing in clinical usage by McMillan and Wolferth,[6] was seldom exhibited in the spontaneously beating turtle heart. In no auricular preparation and in only one ventricular strip did extrasystoles first appear after barium. But when ventricular strips were rhythmically driven by condenser shocks following the addition of barium, there commonly appeared extrasystoles and rapid spontaneous rhythms whose rates were often faster than obtainable by driving before barium was used.

The primary effect of barium on contractility could not be satisfactorily studied because of difficulty in maintaining the necessary constant rate. In such preparations of cold-blooded heart the size of the beat decreases when the rate rises. Those observations that were made indicated a somewhat inconstant effect. At times there occurred a slight increase in strength of beat, even for a faster rate, but the late effect, from twenty to thirty minutes after

Fig. 1.—To illustrate the effect of barium chloride on the same strip of ventricular muscle on three successive days. Time above kymogram; rate below.

the addition of the drug, was usually a definite decline in beat. In one of the auricles a decrease in beat was caused by a concentration of 1:100,000, which did not effect the rate. The ventricular strips usually showed a decrease in strength of beat independent of rate change. When the bath containing barium was replaced by fresh Ringer's solution, only partial recovery occurred within a short time. However, barium did not cause irreversible impairment of contractility, for when the tissue was tested after twenty-four or more hours, a good beat was recorded. This is well shown in Fig. 1, which represents a ventricular strip that was subjected to barium on three successive days; the tissue was kept in the cold room when not under observation, and the lever tension was constant throughout the period. The greater effect of barium on succeeding days that has been described in connection with its enhancement of rhythmicity was not observed on contractility.

Measurement of the refractory period and of conductivity by electrograms from ventricular strips was rendered difficult because of inability to maintain the tissue at a constant rate of rhythmic stimulation before and after barium. For example, in one experiment the muscle would not follow the usual driving rate of 24 per minute; a rate of 14 could be maintained, but after barium 1:100,000, a spontaneous rhythm of approximately 15 developed, and when barium 1:50,000 was added, the rate rose to 35.7 per minute. According to Sollmann,[1] barium increases excitability of heart muscle. In the few experiments for which satisfactory measurements could be made, the state of excitation, as indicated by the refractory period and measured by the duration of the Q-T interval, was little altered. Change, when present, was usually toward a shortened interval, but the maximum shortening observed was less than 5 per cent (Table I).

TABLE I. EFFECT OF BARIUM ON REFRACTORY PERIOD AND CONDUCTION

EXPERIMENT	D. R.	S. R.	Q-T	Q-Q	BARIUM	Q-T	Q-Q	S. R.
A	24.2	8.5	1.26	.04	1:50,000	1.36	.04	27.0
B	24.2	16.7	1.4	.30	1:100,000	1.34	.28	23.1
	24.2		1.4	.20	1:50,000	1.36	.27	23.1
C	24.2		1.8	.38	1:100,000	1.8	.42	
	24.2		1.78	.42	1:50,000		.32	
D	24.2		1.48	.32	1:100,000	1.44	.32	
	24.2		1.40	.34	1:50,000	1.34	.36	
E	24.2		0.92	.24	1:100,000	1.0	.20	
	24.2		1.0	.05	1:50,000	1.0	.06	

D. R., Rate of rhythmic stimulation.
S. R., Rate of spontaneous beating.
Q-T, Refractory period.
Q-Q, Conduction time between receiving electrodes.

In the normally beating dog heart Rothberger and Winterberg[4] found barium to have no appreciable effect on A-V conduction. Megibow and Katz,[7] who maintained auricular fibrillation in the dog heart, reported that barium chloride enhanced A-V conduction and that the effect was marked and prolonged. In the strips of turtle ventricle, with one exception, conduction effects were slight, and the direction was not constant (Table I). In these experiments the effect on both refractory period and conductivity was minimal.

SUMMARY

The effect of barium chloride on the whole auricle and on strips of ventricle of the turtle heart has been studied. The principal action of the drug was

to increase rhythmicity. This effect was more marked when the rate of beating was relatively low, and also when the tissue had been removed from the animal for twenty-four or more hours. Contractility was usually depressed. The influence on excitability, measured by the Q-T of the electrogram, and on muscle fiber conduction was slight.

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ON THE USE OF THEOPHYLLINE AMINOISOBUTANOL IN ANGINA PECTORIS

Franz Steinberg, M.D., and Julius Jensen, M.D.
St. Louis, Mo.

INTRODUCTION

THE use of theophylline and its derivatives in the treatment of coronary disease has persisted for many years in spite of lack of conclusive evidence regarding its efficacy. There is no doubt that, given intravenously, it has a marked effect upon the myocardium and upon the coronary circulation. It has further been assumed that a similar effect could be obtained by oral administration. In recent years, Smith and co-workers[1] and Riseman[2] have defended the view that theophylline given routinely by mouth helps in the treatment of angina pectoris. However, Smith's data were not adequately controlled and Riseman's covered a wide field of drugs. His results were given in general terms without details. In only one chart is plotted the effect of theobromine upon the exercise tolerance of one patient. The conclusions of these authors have to some extent been based upon a subjective feeling of improvement in the patients. No attempt has been made to discount the stimulating effect of theophylline upon the central nervous system. None of these observations equal in care those published by Evans and Hoyle[3] in 1934. The technique of these authors seemed to us the best available and we have employed it in the present study. Evans and Hoyle failed to demonstrate a decrease of the number or severity of the attacks of angina pectoris in patients

From the Department of Medicine, Washington University School of Medicine, and the St. Louis City Hospital, Max C. Starkloff Memorial.
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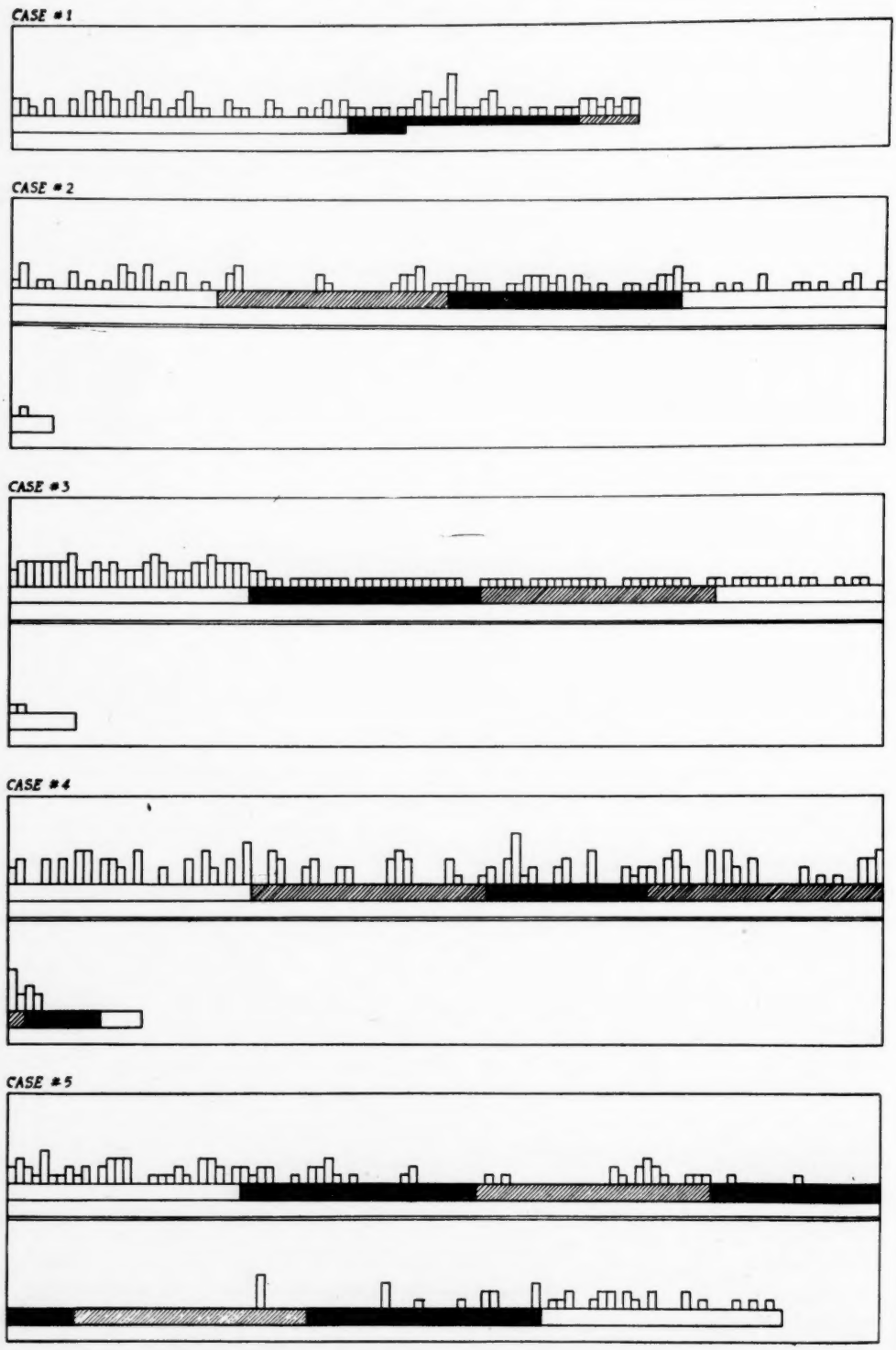

Fig. 1.—Each column represents one day; the height represents the number of attacks recorded, each square indicating one attack. The base band indicates medication: white, no medication; black, theophylline aminoisobutanal; gray, placebo.

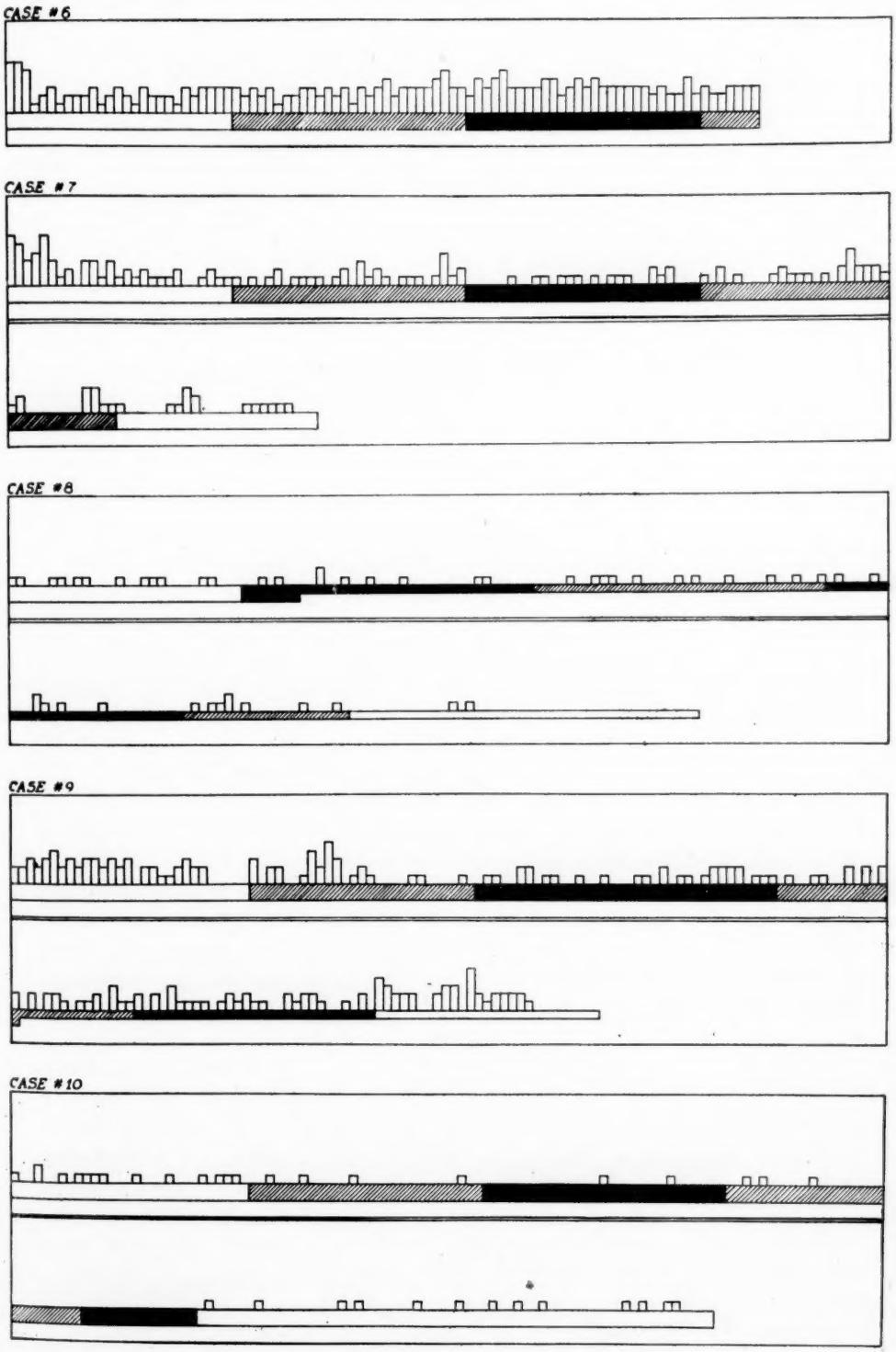

Fig. 1 (Cont'd).—*See opposite page for legend.*

taking theophylline. In 1943, Boyer[4] reviewed the literature on the subject and agreed with Evans and Hoyle that, given orally, theophylline is without effect upon the coronary circulation.

It had been suggested that the reason for this failure to obtain demonstrable effects by oral administration was insufficient dosage, for larger doses cause gastric irritation. Enteric coating does not solve the problem, for tablets so treated may pass undissolved through the intestine. Attempts have been made to produce theophylline preparations which do not upset the stomach. Theophylline aminoisobutanol* is one such preparation. It contains 67 per cent theophylline.

Theophylline aminoisobutanol was given by mouth by Dr. Drew Petersen, on our service, in doses up to 6 gr. three times a day. While good tolerance was not universally observed, the drug was tolerated well enough to be used for an extended study of its effect on ambulatory patients with angina pectoris.

Because theophylline continues to be, more or less wholeheartedly, recommended for oral administration in coronary disease, because it is so widely used in actual practice, and because it was thought that perhaps the Wm. S. Merrell Company had overcome the principal objection to its oral use, it was decided once more to test theophylline in general and specifically the new derivative, theophylline aminoisobutanol.

TECHNIQUE

The technique used by Evans and Hoyle was closely followed. The patients kept diaries of their attacks and the time of observation was divided into periods of four weeks each. After a preliminary period of observation, periods of taking theophylline aminoisobutanol and periods when the patients received a similar but inert tablet followed in irregular sequence.

Sixteen patients with angina pectoris were selected for this observation. Ten of these continued under observation for from twelve to twenty-four weeks. Only in these were the findings considered sufficiently complete to allow conclusions. Nine of them had arteriosclerotic heart disease and one had syphilitic heart disease, which probably caused his angina.

The diagnosis of angina pectoris was established by a carefully taken history, emphasis being placed upon the relation of the pain to effort, emotion, meals, or weather and on the effect of glyceryl trinitrite. If a myocardial infarction had occurred within six months, the patient was not accepted. The patient was given a complete physical examination before he was accepted and electrocardiograms were taken repeatedly during the period of observation.

The patients were seen weekly by one of us (F. S.). They recorded their attacks on a special form and graded them 1 to 4 on the basis of severity and duration. They also kept count of the number of glyceryl trinitrite tablets used per week and, finally, they were asked their subjective impressions of the week as a whole, especially regarding factors which might influence their attacks (emotional factors, unusual efforts, intercurrent illnesses, changes of weather).

During the entire period the patients were instructed to use glyceryl trinitrite as needed and a few who had previously taken phenobarbital routinely continued to do so. After four (in one case three) weeks of observation, some of the patients were given theophylline aminoisobutanol, .18 Gm. three times a

* *Produced by W. S. Merrell Co.

day; the others received as many inert tablets of similar appearance. Every four weeks the drugs were changed without the patient's knowledge so that those who had received the drug were now given the inert substitute and vice versa. Three patients who complained of nervousness and nausea (in one case while the placebo was being given) had their dosage reduced by one-half. Finally, in five of the cases, the test periods were followed by additional weeks of observation, during which the patients received neither theophylline aminoisobutanol nor its inert substitute.

Despite all precautions the evaluation of our results proved very difficult. As is well known, angina pectoris is subject to considerable spontaneous variations and is easily influenced by effort, emotion, and other factors, the exact effect of which it is difficult to define in any given case. Furthermore, many patients with angina pectoris are easily influenced and the mere increased attention which they received made them feel better. Some were overanxious to report improvement and would ignore minor attacks, such as they had formerly recorded. All of these means of investigation, therefore, proved unreliable. We found that even the amount of pain which induced the patients to take glyceryl trinitrite tablets varied so much that the number of such tablets taken proved no reliable guide to the effect of our theophylline therapy.

We finally concluded that the number of recorded attacks of angina pectoris was the best indication of such effect. These data were so arranged as to bring out any contrast between the periods when active and inactive drugs were administered. On squared paper a base band was drawn; the space was white when no drug was given, black when theophylline aminoisobutanol was given, and gray when the inert substitute was given. All days of observations were indicated by spaces on this band. On each day one definite attack was indicated by a square, the number of squares indicating the number of attacks on any given day. Such graphic representations of the ten cases which were studied for from eleven to twenty-four weeks are reproduced in Fig. 1.

CONCLUSIONS

It will readily be seen that there is no significant absence of attacks of angina pectoris during the periods when the patients received theophylline aminoisobutanol as contrasted with the periods when they received the inert substance or no drug at all. It is concluded that theophylline in general, and specifically theophylline aminoisobutanol, exerts no demonstrable effect upon the incidence of attacks in patients with angina pectoris.

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DIMINUTION OF EPINEPHRINE SENSITIVITY OF THE NORMAL HUMAN HEART THROUGH THIOURACIL

W. RAAB, M.D.
BURLINGTON, VT.

THE outstanding role played by excessively secreted epinephrine, sympathin, and related catechol compounds as myocardium-anoxiating and myocardium-damaging agents in the pathogenesis of various forms of heart disease, such as angina pectoris, myocardial degeneration, uremic heart, and sudden cardiac death,[1a-d] makes it desirable to protect the heart muscle against the continually or paroxysmally exerted toxic chemical effects of the sympathomimetic amines named. This can be attempted either by reducing exaggerated adrenal medullary secretion through x-ray irradiation of the adrenal region[2] or by removal of an adrenal medullary tumor or, on the other hand, by artificial diminution of the sensitivity of the heart to epinephrine.

A marked intensification of the stimulating and potentially damaging effects of epinephrine on the heart through the thyroid hormone is a well-established fact.[1e, 3-9] Thyroidectomy produces the opposite situation: the cardiac effects of epinephrine are diminished.[6, 10-12] The beneficial results of thyroidectomy in angina pectoris and heart failure[13, 14] are, accordingly, essentially attributed to the elimination of the thyroid hormone as a factor which sensitizes the heart muscle to the anoxiating action of secreted epinephrine and sympathin.[1a, 11, 12]

It was to be anticipated that thiouracil treatment, by suppressing thyroid secretion,[15] would yield analogous results, and both experimental and clinical observations corroborate this assumption. In rats the resistance of the heart muscle to injected toxic doses of epinephrine and to excessive myocardial epinephrine accumulation was found to be markedly increased after administration of thiouracil,[1e] and striking therapeutic results were obtained in patients with angina pectoris through treatment with thiouracil, while the symptoms reappeared when placebos were substituted for the drug without the patient's knowledge.[1f]

The following observations were carried out in order to ascertain the diminishing effect of thiouracil on the epinephrine sensitivity of the normal human heart.

METHOD

Ten physically healthy young male patients of the Vermont State Hospital were subjected to thiouracil medication for three months (daily total dose, 0.4 Gm. in two portions*). Before and at the end of the medication period the following tests were obtained: basal metabolic rate, blood pressure, pulse rate, and electrocardiogram at rest; electrocardiogram immediately after exercise (36 times hopping on one foot); blood pressure and pulse rate after subcutaneous

From the University of Vermont, Medical College, Department of Medicine, Burlington, Vt., and the Vermont State Hospital, Waterbury, Vt.

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injection of from 0.5 to 1.0 mg. of epinephrine during thirty minutes, measured at six-minute intervals; electrocardiogram taken twelve and thirty minutes after injection.

The psychiatric diagnoses of the test subjects were dementia praecox and psychoses with mental deficiency. While the patients were fairly cooperative as a whole, it was not possible in some of them to establish ideal standard conditions for basal metabolism determination and the readings, therefore, must be regarded with caution.

No unpleasant side reactions were complained of after epinephrine injection; there was some pallor but no trembling, no intense palpitation, and no precordial distress. The thiouracil tablets were well tolerated. Weekly leucocyte counts showed normal values without exception. There developed no clinical features of hypothyroidism.

RESULTS

Basal Metabolic Rate.—Marked decreases below the zero line occurred in only two patients (Cases 7 and 9). In four other patients there were minor decreases. The increases in two patients (Cases 3 and 10) may be explained by the distinctly changed mental state of these subjects at the times of the determinations.

Blood Pressure and Pulse Rate.—Changes of the blood pressure level observed after thiouracil medication were rather irregular (Tables I and II), but there was a slight decrease of the average systolic and diastolic pressure. The same applies to the pulse rate at rest.

TABLE I

CASE	AGE (YR.)	B.M.R.		BLOOD PRESSURE AT REST				PULSE RATE AT REST		AVERAGE* REACTION TO EPINEPHRINE INJECTION					
				SYSTOLIC		DIASTOLIC				BLOOD PRESSURE				PULSE RATE	
										SYSTOLIC		DIASTOLIC			
		BEFORE THIOURACIL	AFTER THIOURACIL	BEFORE THIOURACIL	AFTER THIOURACIL	BEFORE THIOURACIL	AFTER THIOURACIL	BEFORE THIOURACIL	AFTER THIOURACIL	BEFORE THIOURACIL	AFTER THIOURACIL	BEFORE THIOURACIL	AFTER THIOURACIL	BEFORE THIOURACIL	AFTER THIOURACIL
1	27	+ 3%	+12%	122	112	84	64	76	80	+11	+21	−21	−15	+10	+ 1
2	38	− 1%	− 5%	122	120	68	66	68	64	+ 7	+ 9	− 5	− 8	+10	+ 9
3	32	−14%	+ 5%†	130	126	68	78	72	54	+32	+23	−48	−33	+ 6	− 2
4	37	+20%	+14%	130	138	72	80	76	76	− 6	+ 5	− 2	−15	+ 4	− 2
5	25	+12%	± 0%	116	106	82	58	76	78	+ 4	+ 4	−12	−15	+10	− 4
6	22	+ 9%	+ 5%	116	106	68	68	72	64	+10	+10	− 9	−10	+12	− 7
7	22	− 3%	−19%	128	112	78	86	96	92	+ 4	± 0	−28	+ 3	+ 9	− 3
8	34	+ 6%	+ 6%	134	140	90	86	98	90	+ 5	+ 6	− 9	−16	+ 5	−10
9	23	+ 1%	−23%	148	156	80	90	88	88	+ 6	+15	−22	−23	+ 1	+10
10	23	−18%‡	− 4%‡	108	98	68	32	66	52	+ 9	+ 5	−12	+ 3	+ 4	+ 2

*Average of five readings taken during thirty minutes after injection of 0.5 mg. of epinephrine (Cases 5, 6, and 10) and of 1 mg. in the other seven patients.

†Tense and restless.

‡Depressed and mute at time of first determination; rather excited at the time of the second determination.

Also, the reactions of the blood pressure to epinephrine injection were only slightly and uncharacteristically altered after thiouracil medication. However, the epinephrine-induced acceleration of the heart was diminished throughout after thiouracil with only one exception, and in six patients the pulse rate was even decreased after epinephrine injection following thiouracil treatment.

Electrocardiogram.—At the end of the thiouracil period there were a few rather irregular changes of the resting electrocardiogram as compared with the original details. In two patients there was an inversion of the T wave (T_1 in

TABLE II. AVERAGE VALUES OF TEN PATIENTS BEFORE AND AFTER THREE MONTHS OF TREATMENT WITH THIOURACIL (DAILY, 0.4 GM.)

	BEFORE THIOURACIL	AFTER THIOURACIL	DIFFERENCE
Basal metabolic rate	+1.5%	−1.0%	−2.5%
Body weight	152 lb.	152 lb.	±0 lb.
Systolic blood pressure	125 mm.	121 mm.	−4 mm.
Diastolic blood pressure	76 mm.	71 mm.	−5 mm.
Pulse pressure	49 mm.	50 mm.	+1 mm.
Pulse rate per minute	79 beats	74 beats	−5 beats
Response to subcutaneous injection of epinephrine			
Elevation of systolic blood pressure*	8 mm.	10 mm.	+2 mm.
Fall of diastolic blood pressure*	17 mm.	13 mm.	−4 mm.
Increase of pulse pressure*	26 mm.	24 mm.	−2 mm.
Change of pulse rate	+7 beats	−1 beat	−8 beats

*Total average of the individual averages of five readings taken at six-minute intervals during thirty minutes following injection in each patient.

Case 7, T_2 and T_3 in Case 8). T_4 was slightly lowered in eight patients; the T waves in other leads were slightly lowered or slightly increased in a minority of instances. No low voltage of the myxedematous type was seen in any patient.

Following physical exercise only four persons (Cases 5, 7, 9, and 10) showed appreciable alterations of the electrocardiogram (lowering of the T wave in one to three leads) prior to thiouracil treatment. After thiouracil treatment these reactions were only slightly modified (Table III), but with an average diminution of the depression of T_1 and T_4.

TABLE III. DIFFERENCES BETWEEN THE ALTERATIONS OF THE T WAVES DUE TO PHYSICAL EXERCISE BEFORE AND AFTER THIOURACIL (MILLIMETERS)

CASE	T_1	T_2	T_3	T_4
5	−1.0	0	−0.5	−5.0
7	−0.5	0	0	+0.5
9	−1.0	0	+0.5	+1.5
10	−0.5	0	0	−1.5
AVERAGE	−0.8	0	0	−1.1

Epinephrine injections before thiouracil were followed by alterations of the T wave in two or more leads in most patients (lowering in twenty-seven instances, increase in nine, and no change in four). After thiouracil treatment these electrocardiographic responses to epinephrine were slightly to markedly weakened in all subjects (Table IV), the depression of the T waves being diminished or entirely abolished. Prethiouracil inversions of T_1, T_3, and T_4

TABLE IV. DIFFERENCES BETWEEN THE ALTERATIONS OF THE T WAVES DUE TO EPINEPHRINE (TWELVE AND THIRTY MINUTES AFTER INJECTION) BEFORE AND AFTER THIOURACIL (MILLIMETERS)

	T_1		T_2		T_3		T_4	
CASE	12 MIN.	30 MIN.	12 MIN.	30 MIN.	12 MIN.	30 MIN.	12 MIN.	30 MIN.
1	−1.0	−1.0	−1.0	−0.5	0	0	−4.0	−3.5
2	0	0	+0.5	+0.5	−0.5	−0.5	−1.5	−1.5
3	−1.0	−1.0	−1.0	−1.5	−0.5	−0.5	−1.5	−3.0
4	0	−0.5	−0.5	−0.5	−0.5	0	−1.0	−3.5
5	−1.5	−2.0	+0.5	0	−0.5	−1.0	−4.0	−4.0
6	−1.5	−1.0	−4.0	−4.5	−7.5	−7.5	−2.5	−0.5
7	−1.0	−1.0	−0.5	0	−0.5	−0.5	−2.5	−1.0
8	+0.5	0	−0.5	0	0	−0.5	−1.0	−0.5
9	−0.5	−0.5	0	0	0	0	−2.0	−1.5
10	−1.0		−0.5		−1.0		−0.5	
Average	−0.70	−0.77	−0.70	−0.72	−1.1	−1.2	−2.1	−2.1

due to epinephrine (Cases 6 and 7) were absent after thiouracil treatment. Only in four of seventy-six individual single leads was there a very slight (0.5 mm.) elevation of T waves compared with the corresponding leads before thiouracil. The averages (Table IV) show a diminished response of the T waves to epinephrine in all leads after thiouracil treatment both twelve and thirty minutes after injection.

DISCUSSION

The irregular and altogether insignificant changes of the systolic and diastolic blood pressure and of their response to epinephrine injection following inhibition of thyroid function through three months of thiouracil treatment are essentially in agreement with similar results obtained by Riseman, Gilligan, and Blumgart[16] in man after total thyroidectomy.

The resting pulse rate did not show any characteristic alteration after thiouracil, but the epinephrine-induced cardiac acceleration was regularly diminished with only one exception, and in more than half of the patients it was even transformed into a retardation. No clear relationship was observed between this behavior of the heart rate and the degree of response of the basal metabolic rate to thiouracil. A rather wide individual range of the latter has also been noted by other observers[15] in thyrotoxic patients and by me[1f] in ten nonthyrotoxic patients with angina pectoris.

According to Riseman and co-workers,[16] the "cardiovascular sensitivity" to epinephrine (not including the electrocardiogram) remains unchanged after thyroidectomy unless the basal metabolic rate falls below –30 per cent. Levels as low as this were not reached in the thiouracil studies discussed herewith. Eppinger and Levine[12] observed a diminution of the blood pressure and pulse reactions to epinephrine soon after thyroidectomy but not regularly. Leblond and Hoff[17a] found the heart rate and heart size of thiourea-treated rats diminished. DiPalma and Dreyer[18] did not notice any change of the effect of epinephrine on the intestinal movements of three rats following from one to three weeks of treatment with thiourea. Cardiovascular reactions were not included in these experiments, however. Acute single administrations of thiourea were ineffective regarding the response of blood pressure and pulse rate to epinephrine as one would expect in view of the slowness with which thiourea leads to a hormone depletion of the thyroid gland.

From the foregoing discussion of personal observations with thiouracil it would appear that the epinephrine sensitivity of the heart is much more affected by reduction of the thyroid function than that of the peripheral vascular system. This is demonstrated not only by the almost exceptionless diminution of the heart rate reaction, but also by the weakening or abolition of the electrocardiographic effects of secreted (exercise test) and injected epinephrine after thiouracil in all cases. Numerous observations[1a] have shown that the electrocardiogram is a sensitive criterion for the epinephrine-induced myocardial anoxia.

The heart muscle possesses an outstanding tendency to accumulate injected epinephrine.[1a] The epinephrine tolerance-decreasing effect of thyroxine as well as the epinephrine tolerance-increasing effect of thiouracil, which renders otherwise inevitably fatal myocardial epinephrine concentrations innocuous,[1e] are in keeping with the conception of an indirectly protective effect of thiouracil on the heart through elimination of the epinephrine-potentiating thyroid hormone.

Striking therapeutic results obtained by me[1f] in angina pectoris patients with thiouracil therapy and the normalization of the anoxic electrocardiogram in some of these patients were achieved and interpreted on the basis of this same rationale. They seem to contradict the conclusion of Riseman and co-workers[16] that the therapeutic effect of thyroidectomy in angina pectoris is mainly due to the mechanical interruption of sensory impulses from the heart to the central nervous system. On the other hand, they corroborate the opinion of Eppinger and Levine,[12] Shambaugh and Cutler,[11] and Raab[1f] that thyroidectomy improves the anginal symptoms by reducing the sensitivity of the heart to secreted epinephrine.

The blood pressure of patients with angina treated with thiouracil remained unchanged. It is not directly involved in the mechanism of angina pectoris.

Neither in patients with angina nor in the physically normal persons examined in this study was there a strict parallelism between the response to thiouracil of the basal metabolic rate on one side and of the cardiac condition on the other. Disappearance of the anginal symptoms followed the basal metabolic rate much more closely than was the case regarding the epinephrine resistance of the electrocardiogram. This apparent divergence may be explained by the questionable accuracy of the basal metabolism readings in some of the mentally abnormal and, in part, slightly tense subjects used for the latter observations. The conclusion may be justified, however, that the fall of the basal metabolic rate is just one manifestation of reduced thyroid activity and that the diminished epinephrine sensitivity of the heart is a separate one, not directly dependent on the degree of reduction of general oxygen consumption of the body. The statement of Leblond and Hoff[17b] that the effect of thyroxine on the heart is a direct one and not the consequence of general metabolic stimulation is in agreement with this view.

CONCLUSION

Thiouracil administration for three months to ten physically healthy persons was followed by a significant diminution of the sensitivity of the heart to epinephrine (heart rate, electrocardiogram).

The behavior of the blood pressure was rather uncharacteristic.

Implications of these observations regarding the pathogenesis and treatment of angina pectoris are briefly discussed.

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FATAL AGRANULOCYTOSIS OCCURRING DURING TREATMENT WITH THIOURACIL

PAUL H. WOSIKA, M.D., PH.D., AND LADISLAUS BRAUN, M.D.
CHICAGO, ILL.

TOXIC reactions during the course of thiouracil therapy have been reported. Their incidence is about 10 per cent.[1] Most of these are mild and disappear when the drug is discontinued or its dose reduced. Unfortunately, cases of agranulocytosis have been observed as a complication, and practically every publication stresses the possibility of its occurrence. Some of these cases have ended fatally. The necessity of continuous clinical and laboratory studies, the repeatedly stressed fatalities, and probably the unavailability of the drug are factors responsible for the extremely cautious attitude toward thiouracil. In our experience a great majority of the profession voices the opinion that "many deaths" have occurred during its experimental trial. Some statements in the literature contribute to this belief, but there is confusion concerning fatalities.

The first case of agranulocytosis occurring during thiouracil therapy was published by Astwood,[2] and although the patient recovered, this case was repeatedly quoted as fatal agranulocytosis.[3, 4] Newcombe and Deane's[5] case, in which the patient recovered, was referred to similarly. In Himsworth's[3] own case of agranulocytosis, the patient's blood count had become elevated (26,000

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From the Departments of Medicine of the Illinois Masonic Hospital and The Chicago Medical School.
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white blood cells; 76 per cent neutrophiles) when the patient contracted bronchopneumonia and died. A "third fatal case"[3] was mentioned but details were not given.

Actually, there were no cases of fatal agranulocytosis published in detail before the reports of Kahn and Stock,[6] Ferrer, Spain, and Cathcart,[4] and Gargill and Lesses.[7] We are now reporting a fourth fatal case occurring during treatment with thiouracil albeit this tends to emphasize the fatalities.

CASE REPORT

The patient, a 50-year-old widow, was referred for care of hyperthyroidism. The family history revealed that the mother died of a goiter and heart trouble at 54 years of age. One sister had hyperthyroidism. The past history revealed that the patient had had three full-term uneventful pregnancies. At 40 years of age (1934) she developed peritonitis following an abortion and was hospitalized for three months. In 1940 an incisional hernia was repaired. She was hospitalized in February, 1943, for an upper respiratory infection, for which she received approximately 22.0 Gm. of sulfathiazole without incident. Menopausal symptoms began in this year (age, 49) and she was treated with estrogenic substance. This improved a mild neurodermatitis that had proved resistant to various dermatologic therapeutic measures. We are unable to state whether or not this represented some form of allergy. In March, 1944, she weighed 162 pounds, and since she was only 60 inches tall, she sought medical care for reduction in weight. Her physician prescribed amphetamine sulfate. No basal metabolic rate was determined. She lost 16 pounds on this management and in July noticed that she was nervous and that the hot flashes recurred. Friends noted a protrusion of her right eye, for which she consulted her ophthalmologist (Dr. M. H. Cottle), who referred her for care of the hyperthyroidism.

The physical examination revealed a slightly obese woman with a warm moist skin and tachycardia. The thyroid was symmetrically enlarged and smooth. The blood pressure was 150/90 mm. Hg. There was a long midline suprapubic scar and a few lower extremity varicosities. Right unilateral exophthalmos was present.

Laboratory studies revealed plus 43 to plus 85 basal metabolic rates; 132 mg. per cent blood serum cholesterol; normal blood count and urine. She was given 0.6 Gm. thiouracil* for thirty-one days, 0.4 Gm. daily for fourteen days, 0.2 Gm. for twenty-one days, and 0.1 Gm. for ten days; the total dose was 29.4 Gm. The basal metabolic rate was reduced markedly after six weeks, the pulse rate became slower, and no change was noted in the blood count or weight. The exophthalmos and general condition improved dramatically.

The patient was seen November 13 when her throat was slightly injected and her temperature was 99.6° F. The lungs were clear. Thiouracil was stopped. The throat condition became worse, until on November 15 swelling, marked redness, and a dirty white exudate were observed. The temperature was 103° F. She had become toxic and restless. She was hospitalized and the blood count showed 84 per cent hemoglobin (Haden-Hausser), 4,980,000 red blood cells per cubic millimeter and 2,500 white blood cells. No neutrophiles were found in the film. Mild jaundice was noted. The white count upon successive days was 3,000, 1,200, and 500. The final differential count (consultation by Dr. R. Isaacs) was polymorphonuclears, 4; polymorphonuclears, young, 2; lymphocytes, 59; monocytes, 18; endothelial cells, 10; plasma cells, 7.

The patient died November 19 with a temperature of 106° F. after a deepening coma in spite of receiving, daily, 40 c.c. of pentnucleotide intramuscularly, 6 c.c. yellow bone marrow, 500 mg. ascorbic acid, 100 mg. thiamine chloride, a transfusion of 500 c.c. of whole blood, and 10 units of crude liver extract intramuscularly. She also received a few oral 2 c.c. doses of folic acid and 15,000 units of penicillin every two hours for three days along with continuous oxygen therapy.

The post-mortem examination (performed by Dr. J. P. Simonds) revealed a diffuse large ulceration of the entire pharyngeal wall extending to the pyriform fossae. It was covered by a dirty grayish membrane, the bases being irregular and dark reddish purple in color. The ulceration covered the arytenoepiglottic folds and the internal surface of the epiglottis. The area surrounding the ulcer was swollen.

*The thiouracil used was supplied by the Upjohn Company, Kalamazoo, Mich.

The thyroid weighed 22.5 grams. There was a circumscribed grayish nodule in the left lobe. Sections showed a fairly well encapsulated mass composed of relatively small acini lined with cuboidal epithelium. The small lumina were empty or were filled with a thin homogeneous coagulated material. The stroma near this nodule was thin and structureless. In other areas there were numerous bands of connective tissue stroma that divided the parenchymatous tissue into irregular masses. The acini varied in size and were lined with tall columnar epithelium, forming invaginations into the larger lumina. Colloid was present in very few of these acini. Areas of lymphocytic infiltration were common.

The adrenals were slightly larger than normal with the cortex approximating 3 mm. in thickness. The outer zone was yellow and the inner zone was brown. Microscopically the adrenals showed a marked congestion of the medulla and the inner portion of the cortex. Lymphoid material was not present in the cortex in a normal quantity.

A section of the bone marrow showed the bony trabeculae to be normal. The marrow appeared cellular; many megakaryocytes and cells of the erythroblastic series were present. A few promyelocytes and myelocytes but no mature granulocytes were noted.

DISCUSSION

The connection between chemotherapeutic drugs and agranulocytosis was pointed out by Kracke.[8] With the introduction and wide use of the sulfonamides, the importance of this complication gained new emphasis. The relation between the action of thiourea derivatives and sulfonamides has been noted repeatedly. That thiouracil also may have toxic effects on the bone marrow was learned early in its clinical use. One of the first three patients with hyperthyroidism treated with thiouracil developed agranulocytosis (Astwood[2]). Later experiences differ significantly. McGavack and associates[9] encountered one case of agranulocytosis and three cases of leucopenia among twenty-six patients treated. Astwood[10] more recently reported one case of agranulocytosis and one of leucopenia in a total of sixty-two treated patients. Himsworth[3] reported one instance of agranulocytosis and two of leucopenia among twenty-two patients. Gargill and Lesses[7] observed one fatal case of agranulocytosis and one of leucopenia among forty-four patients. Williams (quoted by Jackson[11]) treated 210 patients and observed agranulocytosis twice. Grollman and Gryte[12] noted four cases of leucopenia developing on the third or fourth day of treatment in eighteen patients. In Rose and McConnell's[13] series of thirty-seven patients two had leucopenia with pharyngitis. Gabrilove and Kert[14] noted leucopenia in one of nine patients. Paschkis and co-workers[15] encountered one case of leucopenia in twenty-one patients. Moore and associates[16] found two cases of leucopenia in fifty-two patients and Bartels[17] only three cases of leucopenia in 119 patients treated preoperatively. Palmer[18] observed nine instances of transitory leucopenia in fifty patients, and Beardwood and Levinson[19] only observed it twice in twenty patients, all of whom required only a temporary discontinuance of the drug. Other authors observed neither agranulocytosis nor leucopenia: Reveno[20] in nine, McGregor[21] in twenty, Watson and Wilcox[22] in eleven, Martin[23] in six, and Nussey[24] in 27.

Nine cases (including the present report) of agranulocytosis have been reported in some detail. Of these, five of the patients have recovered and four have died. These have been summarized in Table I. Six were women and two men, the usual ratio observed in agranulocytosis. The recorded ages were between 37 and 70 years, only one being under the age of 50 years. The length of time thiouracil was administered until agranulocytosis developed was from thirty-five days to slightly more than one year. The daily dose at the time the complication appeared was between 0.1 and 1.0 Gm.; in eight of the

nine patients, it was 0.6 Gm. or less. The total dose of the drug consumed was between 16.8 and 168.3 Gm., but in seven of the nine patients it was less than 50 Gm. In one patient (Case 8) the agranulocytosis appeared during a third course of thiouracil, following rest intervals of four and one-half months and seven weeks. In the other instances the drug was taken without interruption.

TABLE I. SUMMARIZING DATA OF THE PUBLISHED CASES OF AGRANULOCYTOSIS

CASE	REPORTED BY	SEX	AGE (YR.)	DURATION OF TREATMENT	DOSE AT TIME AGRANULOCYTOSIS OCCURRED (GM.)	TOTAL AMOUNT OF THIOURACIL (GM.)	AVERAGE DAILY DOSE (GM.)	OUTCOME
1	Astwood	M	37	35 days	1.0	40	1.14	Recovered
2	McGavack	F	50	42 days	0.5	21	0.5	Recovered
3	Williams	?	?	42 days	0.2	16.8	0.4	Recovered
4	Linsell	F	61	126 days	0.2	49.2	0.39	Recovered
5	Himsworth	F	?	52 days	0.6	64	1.23	Died of bronchopneumonia
6	Kahn and Stock	F	62	54 days	0.4	30.8	0.57	Died
7	Ferrer and coworkers	M	70	5 mo.	0.6	168.3	1.12	Died
8	Gargill and Lesses	F	56	Little over 1 yr.	0.2	49.4		Died
9	Wosika and Braun	F	50	76 days	0.1	29.4	0.38	Died

Thus, on the basis of this small number of reported cases of agranulocytosis, it would seem that the danger of agranulocytosis is apparently increased in patients over 50 years of age. The duration of the treatment, the size of the daily dose, and the total amount of drug consumed are of no determining importance. However, three received an average daily dose which exceeded 1 Gm. daily for the whole period of treatment, which is definitely higher than the dosages used currently. The time of the onset of the agranulocytosis was between five and eight weeks in five of the nine patients, but the danger of the complication still was present after this time. Usually, the first five weeks are considered the most likely period for the development of complications. It should be noted that in none of the published cases did agranulocytosis develop before the end of the fifth week.

The effectiveness of thiouracil would seem to be established. Close supervision of the patient must be maintained throughout the entire period of its use. Evaluation of the toxicity must await the publication of additional cases with detailed data.

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PROGRESS

PROBLEMS OF ERYTHROCYTE DISINTEGRATION WITH PARTICULAR REFERENCE TO THE LIFE SPAN OF THE RED CELL

KARL SINGER, M.D.
BOSTON, MASS.

THE THREE INTERDEPENDENT FACTORS ACTIVE IN MAINTAINING THE NORMAL RED CELL LEVEL

THE number of red cells in the blood is kept fairly constant under physiologic conditions. This constancy is usually explained as being caused by a balance between production of red cells and their release into the circulation and by erythrocyte disintegration. Another factor, often not mentioned, is, however, involved; that is, the lifetime of the erythrocyte in the circulation. If this lifetime is abnormally shortened or prolonged, the rate of production and release must necessarily be altered.

Shortening of the average lifetime of the red cell may be due either to endogenous factors (faulty structure of the erythrocytes, abnormal hemolyzing activities) or may be caused by removal of erythrocytes from the circulation (for example, in hemorrhage), thus abruptly and prematurely terminating their useful life. Only in the endogenous conditions will the clinical signs of increased hemolysis be present.

Prolongation of red cell longevity has so far not been clearly demonstrated; it has been believed to occur after splenectomy.[1, 2]

These three variables, involved in the maintenance of a normal red cell level, are obviously interdependent. In Fig. 1 are visualized the various mechanisms which may upset their balance and also the occurring compensatory reactions. If these compensatory activities are inadequate, an abnormal red cell count will result. For a better understanding of the various types of anemias, particularly the hemolytic syndromes, it will often be valuable to investigate all three factors in any given disorder.

Erythrocyte production and release can be recognized by examining the bone marrow and by enumerating the reticulocytes. It is still a moot question whether all newly delivered red cells are reticulated.[3, 4]

Increased erythrocyte destruction manifests itself by quantitative changes in pigment metabolism. Except for the hemoglobinemias, where hemoglobin is at least partly lost into the urine, the liberated blood pigment is, to a very large extent, transformed into bilirubin. The bile pigment is then converted into various derivatives in the intestines. By means of Watson's method,[5] some of these substances (urobilinogen, stercobilinogen) can be determined quantitatively. As the pigment output stems from the total amount of circulating hemoglobin, great individual variations are found. The normal figures fluctuate between 50 and 200 mg. per twenty-four hours. This wide range of normal values is eliminated by means of the hemolytic index[1]:

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$$\frac{\text{Average pigment output per day}}{\text{Total hemoglobin in grams}} \times 100$$

This index expresses the pigment excretion per day in relationship to 100 Gm. of hemoglobin. The normal range was found to be from 11 to 21 mg. per 100 Gm. of hemoglobin.[1, 2] The total hemoglobin is computed from the hemoglobin in grams per 100 c.c. of blood and from the total blood volume. The blood volume may be either determined directly or computed from Gibson and Evans' formula.[6] The advantage of this hemolytic index is that it takes into account the fluctuations which occur in the pigment excretion with variations in the total mass of hemoglobin in the body. An elevated hemolytic index is not infrequently the only detectable clinical sign of an existing increased rate of hemolysis.

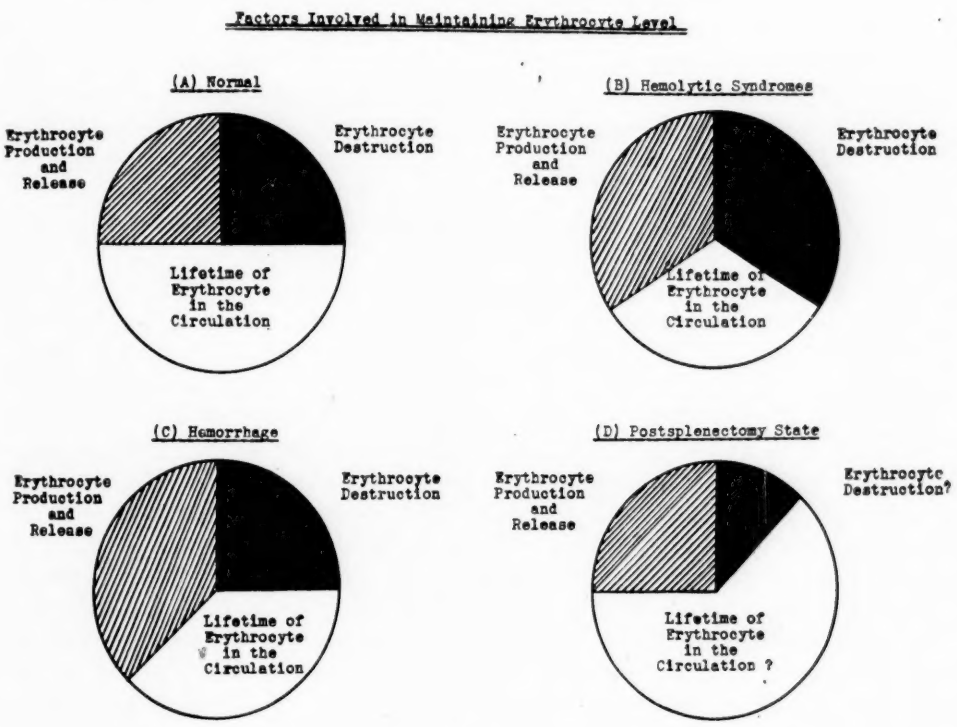


Fig. 1.

Whereas erythrocyte production and destruction are relatively easily measured, determinations of the lifetime of the erythrocytes are much more complicated and are, at present, only of scientific interest. Investigations of this kind have only recently been started. From what is known so far, it is obvious, however, that such determinations promise to become a very valuable tool for the solution of many controversial hematologic problems.

Until recently the longevity of the erythrocytes was often determined by methods not based on physiologic principles; it was believed to be only about 30 days. Today we have sufficient evidence to assume that the true average lifetime is about 100 to 120 days. This value has been established for the dog,[7, 8] the monkey,[9] and the human being by different methods.[10] In dogs with renal biliary fistula which are maintained in perfect health by adding ox bile or a bile salt-vitamin mixture to their diet,[7, 8] it is possible to determine the daily pigment output by measuring the bilirubin in the urine. If in such an

animal a severe anemia is produced by means of acetylphenylhydrazine or by bleeding, one can observe the launching of many young red cells into the circulation within a few days. If the reticulocytes are counted, the peak of the reticulocyte curve may be used as the starting point for the determination of the lifetime of these new cells.[8] During and after the anemia period the bilirubin excretion is first found to be lower than in the control period; this is due to the change in the age distribution of the red cell population, many young cells being present and, therefore, fewer erythrocytes being destroyed. Then gradually there is an increase of the bile pigment output which reaches its maximum after an average of about 110 to 124 days. This peak may be interpreted as being caused by mass disintegration of the red cells which entered the circulation during the repair period of the anemia and which have now reached the end of their lifetime.

In monkeys, approximately the same average lifetime was found by following the daily reticulocyte count after production of a severe anemia.[9] A second reticulocyte peak representing erythrocytes delivered to replace those which entered the circulation at the time of the anemia was observed after from 94 to 117 days. This second peak was used as the end point in the determinations of the life span.

In man, serologic methods of differential agglutination are now mostly employed for determination of the longevity of the erythrocytes. Ashby[11] was the first to transfuse 0 erythrocytes into recipients of different blood groups and to determine the time required for the disappearance of these "foreign" cells. This technique has become more refined by using the agglutinogens M and N for tagging of erythrocytes. Wiener[12] and Mollison and Young[13] were thus able to demonstrate that these transfused red cells survive for from 3 to 4 months in the circulation of a normal recipient. With these serologic methods, the maximal survival time of the donor's erythrocytes is estimated. This survival time, however, is not necessarily identical with the true average lifetime of the red cell. In using the method of differential agglutination, at the starting point (day of transfusion) a mixed population of cells of quite different age is introduced into the recipient. The end point (complete disappearance of the test erythrocytes) is not sharply defined, as the assumption that the youngest cells may survive longest is not self-evident. As Baar and Lloyd[14] remark, red cells produced on any day may vary to some extent in their quality and thus cope differently with the fluctuating forces acting in erythrocyte destruction. Thus the average lifetime may sometimes be appreciably shorter than the maximal survival time.

Recently a mathematical analysis of the results obtained in determining the survival time has been performed by British investigators.[10, 15] These workers found that under strictly physiologic conditions there is a gradual disappearance of the transfused red cells and if the numbers of surviving cells are plotted against time in a diagram, a "linear type of decay" is found. This straight line of disappearance depends on a property (age) of the transfused cells themselves. The normal mode of destruction acts so as to remove in the unit of time a fixed fraction of those cells introduced at transfusion. The straight line of decay is, therefore, the expression of the fact that the erythrocytes are destroyed by normal means. As an average, about 0.83 per cent of the transfused cells are removed from the circulation per day; the true average lifetime was, therefore, again computed to be 120 days.[10] If abnormal conditions exist, the line of disappearance of cells becomes curved, thus indicating

that an additional different hemolytic mechanism may be acting concurrently. Such pathologic mechanisms which do not result in a linear line of decay and which change the straight line into a curved one are probably of such a nature that they destroy the erythrocytes at random, irrespective of their age. If, for instance, Rh-positive cells are transfused into a Rh-negative recipient, the physiologic erythrocyte destruction may be interfered with and accelerated by the developing anti-Rh agglutinins. Increased blood destruction may, theoretically, therefore, be caused either by quantitative increase of an already existing physiologic mechanism or by the concurrent presence of qualitatively different processes which condition the pathologic state.

Lloyd[16] and Dacie and Mollison[17] were the first who transfused pathologic red cells from a patient with hemolytic anemia into normal recipients and also exposed normal cells to the apparently abnormal hemolyzing activities existing in the particular patient. One may call this procedure the method of cross-determination of the lifetime of erythrocytes; in this way it may become possible to decide whether the cause of a hemolytic disorder has to be sought for in a structural defect of a particular type of erythrocyte which is responsible for premature disintegration or whether an abnormal hemolyzing activity is present. Thus the various types of hemolytic syndromes may be quite generally classified as being caused either by the presence of an extra- or an intracorpuscular abnormality, or by a combination of both.

THE SPLEEN AND HEMOLYSIS

Splenectomy is probably the most successful procedure in the treatment of hemolytic disorders. However, clinical observations demonstrate that excessive hemolysis disappears permanently after splenectomy only in familial spherocytic jaundice and acquired hemolytic anemia[18] but that removal of the spleen is without curative effect in the other hemolytic syndromes. Interpretation of these results of splenectomy is difficult because of scarcity of our knowledge of normal splenic function and of the mechanisms involved in the various types of hemolytic anemias. It is usually assumed that the spleen participates physiologically in erythrocyte disintegration and that in the disorders in which splenectomy is effective, a hyperfunction of the spleen (hemolytic hypersplenism) exists.

Several years ago a study was undertaken of the hematologic data collected from patients who had been splenectomized for various disorders.[1] Patients who had been operated upon for traumatic rupture of the spleen or for thrombocytopenic purpura and who had made a complete recovery were of particular interest, as in these patients no primary disease of the erythropoietic apparatus had been present prior to the operation. In Table I are contained the data of eight of these patients.

In all these patients the quantitative aspects of blood production and delivery were quite satisfactory. There was no anemia and the reticulocyte counts were either normal or slightly elevated. Qualitatively, however, erythrocyte production was not a normal one. All these patients showed Howell-Jolly bodies and target cells (leptocytes), that is, red cells, thinner than normal ones and characterized by a decreased fragility to hypotonic saline solutions. Blood destruction seemed to be diminished. The hemolytic index was definitely, and sometimes considerably, lower than normal in seven of the eight patients. This was first interpreted as a confirmation of the hypothesis that the spleen is engaged in the destruction of normal red cells.[1] According to the interde-

TABLE I. HEMATOLOGIC DATA OF CASES SPLENECTOMIZED FOR NONHEMOLYTIC CONDITIONS

CURRENT NUMBER	SEX	AGE (YR.)	DIAGNOSIS	TIME AFTER SPLENECTOMY	HEMOGLOBIN GM.	HEMOGLOBIN PER CENT	R.B.C. (MILLIONS PER C.MM.)	RETICULOCYTES (PER CENT)	HOWELL-JOLLY BODIES (PER CENT)	TARGET CELLS (PER CENT)	HYPOTONIC FRAGILITY % NaCl SOLUTION BEGINS	HYPOTONIC FRAGILITY % NaCl SOLUTION COMPLETE	SERUM BILIRUBIN (MG./100 C.C.)	FECAL UROBILINOGEN (MG./DAY)	HEMOLYTIC INDEX
1	M	24	Traumatic rupture	9 mo.	14.9	96	4.9	0.8	0.4	9.6	.40	.08	0.68	55.0	8.0
2	F	35	Purpura	18 mo.	14.3	92	4.9	1.4	0.7	2.7	.42	.12	0.77	37.5	6.5
3	F	50	Purpura	3 yr.	13.4	86	4.6	1.5	0.5	0.5	.42	.12	0.33	61.4	11.4
4	F	18	Purpura	3 yr.	14.2	91	4.6	0.5	2.2	3.4	.44	.12	0.37	45.8	6.1
5	F	38	Purpura	8 mo.	14.7	95	4.9	1.1	0.9	4.6	.48	.08	0.26	27.8	4.6
6	M	40	Traumatic rupture	15 yr.	15.9	102	5.3	0.8	1.3	0.2	.46	.20	0.50	72.9	8.9
7	M	45	Purpura	7 wk.	13.0	84	4.8	1.9	0.8	2.7	.48	.12	0.32	22.2	3.4
8	F	25	Purpura	4 wk.	13.2	85	5.3	2.2	0.4	2.4	.40	.12	0.30	25.2	5.6
													Average		6.1

pendence of the three factors active in maintaining the normal red cell level, however, the presence of a quantitatively normal production existing simultaneously with diminished destruction could mean only that the lifetime of the erythrocytes in the postsplenectomy state must be prolonged (Fig. 1).

The question arose whether this interpretation could really be the correct one. Was it likely that removal of a normal organ (for instance, after traumatic rupture of the spleen) should lead to the production of erythrocytes which were apparently better equipped to cope with the forces causing disintegration than were normal red cells? In order to solve this problem it was decided to determine the life span of red cells before and after splenectomy. As clinical material suited for this problem was not readily available, the experiments were performed in dogs with renal biliary fistula, using the method of Hawkins and Whipple[7] previously mentioned. These experiments are published in detail elsewhere.[8] As can be seen from Table II, the average life cycle before and after splenectomy was practically identical.

TABLE II. AVERAGE LIFE CYCLE OF THE ERYTHROCYTE BEFORE AND AFTER SPLENECTOMY

DOG	BEFORE SPLENECTOMY (DAYS)	DOG	AFTER SPLENECTOMY (DAYS)
1	115	4	110
2	108	5	96
3	117	6	109
Average	113	Average	105

However, the red cells in the postsplenectomy state were not normal, as numerous target cells showing increased saline resistance were present. These target cells thus have the same lifetime as normal erythrocytes.

The results of these experiments are of significance to two often discussed problems: namely, the role of the spleen in physiologic erythrocyte disintegration,[19-21] and the explanation of the effect of splenectomy only, in some of the

hemolytic disorders.[22] Demonstration of the almost identical average life cycle before and after splenectomy seems to indicate that "active" participation of the spleen in the physiologic breakdown processes of erythrocytes is either negligible or absent or can be entirely compensated by extrasplenic activities. Lauda,[21] in his monograph on the physiology of the spleen, has critically reviewed all the evidence in support of splenic hemolysis. According to this author it is necessary to differentiate between passive and active hemolysis of the spleen. There is no doubt that this organ takes part in removing the debris of red cells which have already disintegrated in the circulation. The spleen functions as the lymph node of the blood. The concept, however, that the spleen actively removes or destroys red cells under physiologic conditions does not withstand rigorous criticism in Lauda's opinion.

Red cells are thought to be destroyed by phagocytosis, fragmentation, and erythrolysis. The latter may possibly be enhanced by the fat content of the food.[23] As the breakdown of red cells continues after splenectomy, it is evident that the presumed participation of the spleen in this mechanism can only be one feature of the general problem of the means and ways of physiologic erythrocyte disintegration. Although erythrophagocytosis has been observed in the spleen, many authorities[21, 24] believe that this mechanism plays hardly any important role in the destruction of red cells, whereas others[55] are still convinced of its significance. Knisely[25] was unable to notice any erythrophagocytosis in the transilluminated living spleen but observed it in the dying animal or after traumatization. Compensatory erythrophagocytosis in other organs belonging to the reticulo-endothelial system is also not demonstrable after splenectomy.[19] Any active influence of the spleen on fragmentation is unknown, and whether tissue or blood lysins (lysolecithin) manufactured in the spleen are of any real significance has not yet been elucidated.[26] There remains only the apparent influence of splenectomy on pigment metabolism as positive proof for a physiologic splenic hemolytic function. The demonstration of a low hemolytic index after splenectomy may, however, require a different explanation.

The values of the hemolytic index in human beings are not based on the determination of bilirubin but on its derivatives. Investigations in the field of pyrrol chemistry during the last years have revealed the great complexity of the various phases of pigment metabolism. In the diagram on the following page are summarized the three main pathways of hemoglobin disintegration.

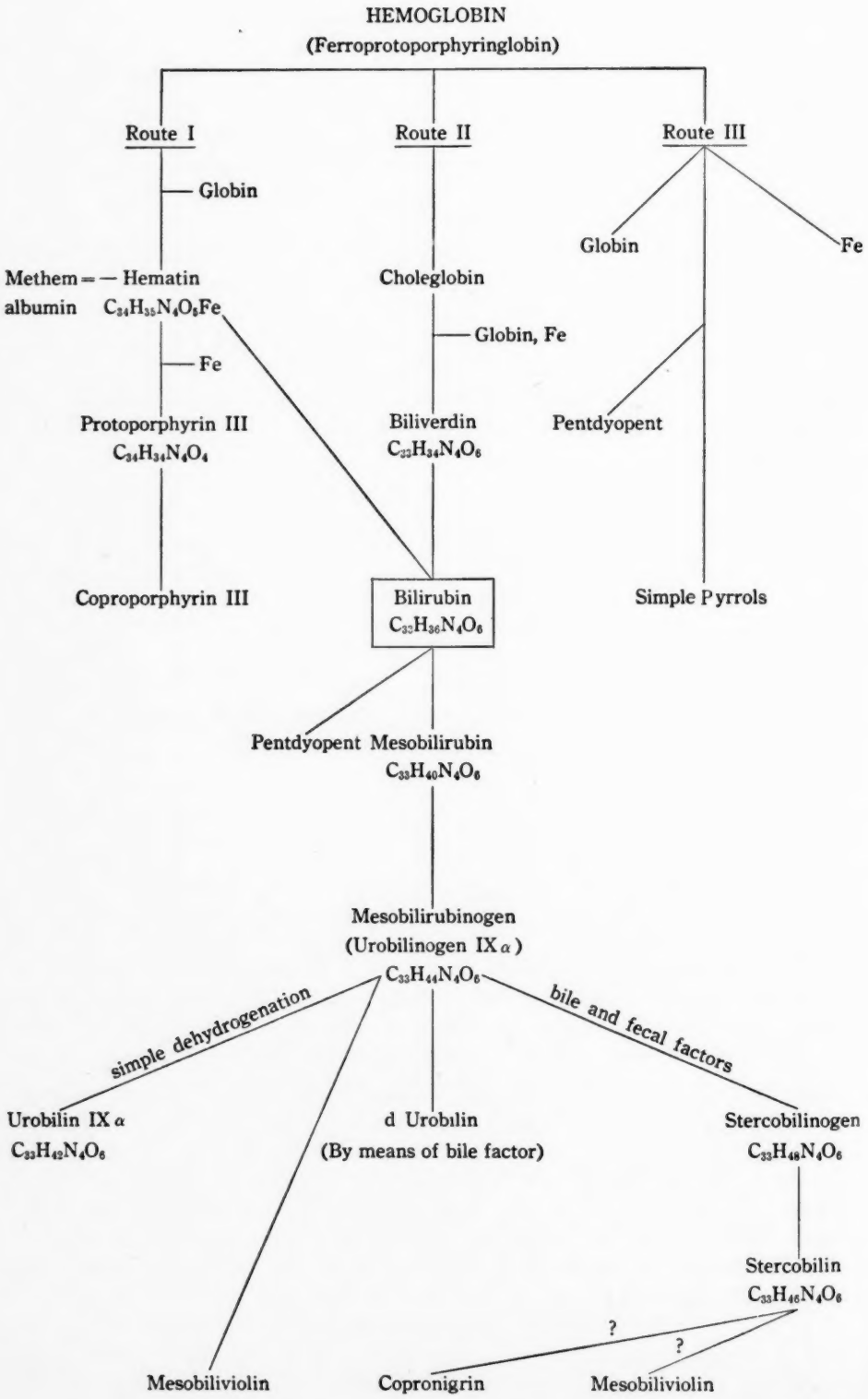
Route I is used when hemoglobin is broken down due to bacterial contamination. First the globin is removed and hematin formed. According to Farley,[27] hematin is immediately combined with the crystal albumin of the plasma to form methemalbumin. Whether hematin can be converted in the organism into bilirubin is still a moot question.[28] * After the iron is split off, protoporphyrin results.

The second pathway is probably used in the body for the breakdown of hemoglobin leading to bilirubin. Lemberg[29, 30] has demonstrated that hemoglobin in the presence of ascorbic acid and molecular oxygen is transformed to choleglobin which has an open porphyrin ring. The first globin- and iron-free pigment is biliverdin, which, however, is rapidly reduced to bilirubin.

Whether the third pathway, where the hemoglobin molecule disintegrates into simple pyrrols or dipyrrols besides iron and globin, has any physiologic significance has not yet been demonstrated. This breakdown was accom-

*Recently Pass, Schwartz, and Watson (J. Clin. Investigation 24: 283, 1945) found a proportional augmentation of fecal pigment after intravenous administration of hematin in human beings. This may be interpreted as evidence of formation of bile pigment from hematin. However, hematin does not seem to be a physiologic precursor of bilirubin.

HEMOLYTIC INDEX
8.0
6.5
11.4
6.1
4.6
8.9
3.4
5.6
6.1


HEMOGLOBIN
(Ferroprotoporphyringlobin)
Route I
Route II
Route III
Globin
Globin
Fe
Methem-albumin
Hematin
$C_{34}H_{35}N_4O_6Fe$
Choleglobin
Globin, Fe
Fe
Protoporphyrin III
$C_{34}H_{34}N_4O_4$
Biliverdin
$C_{33}H_{34}N_4O_6$
Pentdyopent
Coproporphyrin III
Bilirubin
$C_{33}H_{36}N_4O_6$
Simple Pyrrols
Pentdyopent Mesobilirubin
$C_{33}H_{40}N_4O_6$
Mesobilirubinogen
(Urobilinogen IX α)
$C_{33}H_{44}N_4O_6$
simple dehydrogenation
bile and fecal factors
Urobilin IX α
$C_{33}H_{42}N_4O_6$
d Urobilin
(By means of bile factor)
Stercobilinogen
$C_{33}H_{48}N_4O_6$
Stercobilin
$C_{33}H_{46}N_4O_6$
?
?
Mesobiliviolin
Coprongrin
Mesobiliviolin

plished in vitro by Haurowitz and co-workers[31] by means of the vitally important linoleic and linolenic acids in the presence of oxygen.

As hemoglobin is, to a very large extent, quantitatively transformed to bilirubin, determination of this latter pigment should give a reliable indication of the degree of hemoglobin destruction. However, such determinations are not feasible in human beings. Quantitative recovery of bile pigment derivatives is usually employed instead in clinical investigations. Watson's[28] excellent studies have recently demonstrated that the bilirubin derivatives to be encountered in feces and urine are, however, much more numerous than previously believed and that at present they can only partly be quantitatively determined. The results of such investigations of bilirubin derivatives are, therefore, definitely inaccurate. Only if all the bile pigment derivatives stemming from bilirubin could be quantitatively recovered could a truly exact picture of the state of hemoglobin destruction in human beings be obtained. It may be suggested that all derivatives of bilirubin found in the excreta be called "exobilins." The exobilins may then be divided further into (1) bile pigment derivatives which, at the present time, can be quantitatively determined, and (2) into substances, like mesobiliviolin,[32] copronigrin, and probably many others, for which no quantitative methods are yet available. It is obvious that this fact must influence the interpretation of the hemolytic index. An index higher than normal is almost always indicative of an increase of blood destruction; an index lower than normal, however, may be due (1) to diminished bilirubin formation, (2) to a sparing action of the body, or (3) to a change in the ratio normally existing between the quantitatively determinable and nondeterminable exobilins in favor of the latter.[2] It is hypothesized that the lower hemolytic index found after splenectomy may be explained in such a fashion, although no actual evidence for this assumption is available at present. In dogs with renal biliary fistula the bilirubin excretion is not significantly altered by splenectomy.[8]

The possibility that the lifetime of the erythrocyte before and after splenectomy may be identical in dogs only, but not in human beings, does not seem very likely, as the hematologic changes occurring after splenectomy are identical in both species. Further combined studies of the survival time of transfused erythrocytes and the pigment metabolism in splenectomized human beings are necessary to elucidate this problem.

Considering the lack of any positive evidence that under physiologic conditions the spleen participates in erythrocyte disintegration and assuming that in man as well as in dogs the average life span of the erythrocyte after splenectomy is normal, the hypothesis may be advanced that physiologically splenic hemolysis is absent or insignificant. There can be, however, no doubt that under pathological conditions splenic hemolysis does exist. It is selective, conditioned by abnormal properties of the red cells which circulate through the organ.[8]

In familial hemolytic jaundice abnormal erythrocytes (spherocytes) are present which continue to exist after splenectomy. The increased saline fragility of these red cells may stay unaltered or become slightly diminished after removal of the spleen but it still remains within pathologic range.[22] In spite of the continuous presence of abnormally shaped cells, excessive hemolysis ceases abruptly after splenectomy and pigment metabolism becomes entirely normal. The most astonishing feature in this return to normal, often not sufficiently appreciated, is the fact that the extrasplenic "hemolyzing activities" of the body treat the spherocytes in a normal manner. Continuation

of abnormal hemolysis after splenectomy is extremely rare and always accompanied by other atypical features. The spherocytes in familial hemolytic jaundice are, therefore, able to fulfill their function as hemoglobin carriers in the absence of the spleen. Increased function of the spleen, hemolytic hypersplenism, was supposed to explain this phenomenon. Recently, however, cross-determinations of the lifetime of the erythrocytes have rendered this explanation untenable. Lloyd[16] and Dacie and Mollison[17] demonstrated that spherocytes transfused into normal recipients were rapidly destroyed, whereas normal cells transfused into patients with familial hemolytic anemia survived normally. These experiments show that the normal spleen destroys the abnormal cells selectively and that the supposedly pathologic spleen does not attack the normal cells in an abnormal way. No hemolytic "hypersplenism" is, therefore, demonstrable in familial hemolytic jaundice. Some abnormality residing inside the red cells seems to be responsible for their disintegration in or through the spleen.

Not only spherocytes, however, but other abnormal cells as well may be eligible for splenic hemolysis.

Before the discovery of liver treatment, splenectomy was recommended in pernicious anemia. It had no curative effect but was often followed by a transient improvement of the anemia. Eppinger[33] investigated the bile pigment metabolism before and after splenectomy in his patients with pernicious anemia and found a marked diminution of the urobilinogen output after removal of the spleen simultaneously occurring with the improvement of the anemia. Whether these findings are really indicative of splenic hemolysis of the megalocytes requires further studies. That the circulating erythrocytes in pernicious anemia have undoubtedly a much shorter lifetime than normal cells was demonstrated by Morawitz.[34] He transfused normal erythrocytes into a nonsplenectomized case of pernicious anemia to such an extent that the majority of the red cells in the patient's circulation belonged to the donor's group. As the anemia improved for a longer period of time, but no change in the pigment output became noticeable, it is evident that the transfused normal erythrocytes survived much longer than the pathologic megalocytes.

In acquired hemolytic anemia the red cells may or may not be spherocytic and may or may not be more fragile to hypotonic saline than normal ones.[18] Splenectomy often cures these syndromes and if spherocytes are present, they may disappear permanently after removal of the spleen in contradistinction to familial hemolytic jaundice.[35] From the standpoint of our hypothesis, these "splenogenic spherocytes" must first undergo their change in shape in order to become liable for splenic destruction.

In sickle-cell anemia, in the Mediterranean syndromes, and in nocturnal hemoglobinuria, the erythrocytes are also definitely pathologic,[36] but the corpuscular abnormality making them eligible for splenic hemolysis appears to be lacking as can be deduced from the absence of any effect of this operation in these conditions. Only by a better understanding of the nature of the red cell abnormalities which induce splenic hemolysis will this problem be further clarified.

DEFECTS IN THE STRUCTURE OF THE RED CELL AS CAUSES OF HEMOLYTIC ANEMIA

THE DIFFERENTIAL FRAGILITY TEST

We are accustomed to considering the physiologic destruction of erythrocytes as being caused by some "activities" of the body. Be that as it may (and as already pointed out, very little is known about it), the determinations of

the lifetime of the red cells have clearly demonstrated that erythrocyte disintegration also depends on their age. The linear type of decay of transfused cells in man, as noticed by Callender, Powell, and Witts,[10] and the mass disintegration after from 100 to 120 days of red cells launched for the regeneration of an anemia in animals[7-9] are sufficient evidence to support such a statement. Aging of the cell means physicochemical changes in its structure or stroma and apparently if these alterations progress far enough the cells break up. Under pathologic conditions mechanisms may be at work which produce either structural abnormalities in primarily intact cells or red cells which may from the beginning of their life be characterized by certain defects. Such alterations may then lessen their resistance to the vicissitudes of life in the circulation, thus shortening the life span and, therefore, increase the rate of hemolysis. In Table III a classification of the hemolytic syndromes is attempted, based on our present knowledge as to whether extra- or intracorpuscular abnormalities are responsible for the premature disintegration of the cells.

TABLE III. CLASSIFICATION OF HEMOLYTIC SYNDROMES PREDOMINANTLY DUE TO EXTRACORPUSCULAR ABNORMALITIES, INTRACORPUSCULAR ABNORMALITIES, AND UNKNOWN MECHANISMS

A. Extracorpuscular Abnormalities
1. Protozoal and bacterial disease: malaria, bartonella, infections with *Bacillus welchii, Streptococcus hemolyticus* and *viridans*, etc.
2. Chemical agents: phenylhydrazine, arseniureted hydrogen, sulfa drugs, lead, snake venoms, etc.
3. Favism
4. Immune bodies (agglutinins and hemolysins): transfusion accidents, hemolysinic anemias, paroxysmal cold hemoglobinuria, hemolytic anemia of the newborn (erythroblastosis fetalis)
5. Blackwater fever
6. Physical agents: heat, severe burns
B. Intracorpuscular Abnormalities
1. Familial spherocytic hemolytic jaundice
2. Sickle cell anemia
3. Cooley's anemia and the related Mediterranean syndromes
4. Marchiafava-Micheli syndrome: paroxysmal nocturnal hemoglobinuria
5. Pernicious anemia
6. Certain cases of acquired hemolytic anemia responding to splenectomy?
C. Unknown Mechanisms
1. Certain acquired hemolytic anemias of unknown etiology not responding to splenectomy
2. Symptomatic hemolytic anemia occurring in neoplasms, leucemias, Hodgkin's disease, etc.

The common denominator in almost all the conditions classified in Table III as caused by extracorpuscular anomalies is the fact that a great variety of substances of different types can actively injure the red cells. Such an injury (1) may lead to the immediate dissolution of the erythrocytes in the blood stream (hemoglobinemia) or in the organs belonging to the reticulo-endothelial system or (2) it may manifest itself in changes which predispose the red cells to premature disintegration. Aside from the characteristic alterations of the erythrocytes brought about by the malaria plasmodia, certain poisons, for instance, phenylhydrazine, produce the so-called Heinz bodies, highly refractile crystallike aggregates which frequently protrude from the side of the cell.[4] Immune bodies or poisons may also produce spherocytosis with a concomitant increase in saline fragility. Dameshek and Schwartz[37] demonstrated experimentally that spherocytosis develops after injection of anti-red cell sera into animals. Normally the red cell is a biconcave disk. This shape makes it necessary to consider the cell as being in a state of permanent strain

(Ponder[38]). When this strain is released, the cell assumes spherical form as the result of the action of surface tension. What physicochemical changes inside the cell cause this release is unknown. It is obvious, however, that globular shape may often be a consequence of some preceding injury to the erythrocyte. Although spherocytosis is sometimes reversible,[39] usually it has to be considered as a forerunner of hemolysis,[40] possibly in relation to the degree of damage to the cell.

Often classification of a hemolytic syndrome will depend on the result of the cross-determination of the lifetime of the erythrocytes. In patients with blackwater fever transfused normal red cells are as rapidly destroyed as the patient's own cells, a convincing proof for the existence of an extracorpuscular mechanism responsible for the pathologic hemolysis.[41] The nature of this hemolysin is unknown. In severe burns destruction of a considerable volume of erythrocytes may result directly from the heating of blood at the site of the burn. Damage of the red cells manifests itself in fragmentation, budding, and spherocytosis.[54]

In the disorders of Group B, the cause of the hemolytic disease is intracorpuscular without any evidence for the presence of an abnormal injurious agent causing the anomaly. However, some of the abnormalities residing in the red cells have the peculiarity of rendering a particular type of erythrocytes vulnerable to specific physiologic conditions which have no deleterious effect on normal erythrocytes. Ham and Dingle[36] demonstrated that the red cell in paroxysmal nocturnal hemoglobinuria is attacked by a serum factor indistinguishable from serum complement. The spherocyte in familial hemolytic jaundice is eligible for hemolysis in or through the spleen which organ does not alter the lifetime of normal cells. The sickling phenomenon of the red cells in sickle-cell disease depends on the CO_2 tension in their environment, a mechanism which is probably of great importance for the clinical manifestations of this disorder.[42]

It is of great interest that in this group the hereditary hemolytic syndromes can be found. As Haden[43] has emphasized, sickle-cell anemia and Mediterranean anemia are diseases in which there is evidently present an inherited defect in quality of the stroma, so that the cells disintegrate prematurely. The mechanisms which produce these defects are unknown.

The megalocyte in pernicious anemia is a red cell which is also abnormal in structure; the cause of this anomaly is now recognized: it is the absence of the antipernicious principle during the development of the cells in the bone marrow. The lifetime of the pernicious anemia erythrocyte is considerably shorter than that of normal cells[34]; therefore, this disorder should also be classified as a hemolytic anemia.

Only a few years ago spherocytosis was considered to be pathognomonic of congenital hemolytic jaundice. This view had to be abandoned when it was shown that spherocytes could be produced experimentally.[38] Based on these experiments, the hypothesis was advanced that spherocytosis in the hereditary disease may be due to the action of a "hemolysin" or metabolite on normal erythrocytes.[18] This theory, however, cannot be reconciled with the fact that normal cells, transfused into patients with hereditary spherocytic jaundice, have a normal longevity.

The alteration which is present in the spherocytic cells in familial hemolytic jaundice only interferes with their lifetime in the presence of the spleen. The globular shape of the erythrocytes was considered to be the cause of their destruction in the spleen. Whipple[44] believes that normal discoidal cells have

no difficulty in traversing this organ, whereas spherocytes, being thicker, are unable to pass through the stomas of the splenic sinuses. However, clinical experience shows that nonspherocytic cells may sometimes also be hemolyzed in the spleen. Ham and Castle[45] regard intravascular stasis as the immediate mechanism of splenic hemolysis, resulting in increased blood destruction in hemolytic anemias characterized by increased saline fragility of the red cells; they demonstrated that the saline fragility of the erythrocytes in the spleen increased in their experimental animals when prolonged stasis was induced by nembutal anesthesia. As Dacie[22] points out, this hypothesis is also difficult to reconcile with the observation that the greatly engorged spleen in spherocytic jaundice destroys spherocytes selectively. Furthermore, increased blood destruction should be demonstrable in all disorders with enlargement and engorgement of the spleen, which, however, is not borne out by clinical observations.

Haden[46] has admirably correlated the changes in shape of the erythrocytes with the changes in hypotonic fragility. Normal erythrocytes, when placed in hypotonic salt solutions, become progressively more globular due to the entrance of water into the cell until the cell ruptures. Spherocytes, being globular to begin with, already have one of the shapes through which normal cells must pass and, therefore, they are nearer to the "hemolysis point." While there is usually a striking parallelism between resistance of the cell to osmotic hemolysis and the degree of spherocytosis, there are exceptions to this rule as Haden himself emphasized.[43] Occasionally one may come across spherocytosis with normal fragility[40, 43] which may be due to increased resistance of the cell envelope, or one may see little spheroidicity with greatly increased fragility.[43] The physicochemical factors which influence the strength of the cell membrane or the resistance of the stroma to release the strain inside the cell are unknown. However, such deviations from the rule again demonstrate that increased spheroidicity may often be only one of the manifestations of more general cell defects; the latter probably are of much greater pathogenic significance.

That changes in shape and fragility do not necessarily indicate a change in the lifetime expectancy may also be seen from the presence of target cells (leptocytes) with increased saline resistance and a normal lifetime in the postsplenectomy state.[8] Target cells with increased saline resistance are also found in sickle-cell anemia and Cooley's anemia, disorders in which the red cells disintegrate much faster than normally. Recent studies of the members of families in which Cooley's anemia occurred have demonstrated that clinical pictures exist in which numerous target cells are present but without the signs of an increased rate of hemolysis. Transitions may be found to the fully developed, severely hemolytic Mediterranean anemias.[47, 56-58] In these various Mediterranean syndromes, therefore, the leptocytes show a variable tendency to disintegration, which is not directly related to the shape and fragility of the cells. It is believed that the degree of the existing structural defect of these cells conditions their longevity in the circulation.[8*]

Anomalies of red cells may also manifest themselves in a pathologic resistance to agents other than hypotonic, saline solutions. Such studies have been performed with lysolecithin, saponin, and acids, and characteristic reac-

*This belief is supported by recent experiments of Valentine and Neel (Am. J. M. Sc. 209: 741, 1945). These workers showed that target cells may be produced in vitro by suspending normal erythrocytes in plasma or serum rendered hypertonic by the addition of chemicals or by evaporation. Naturally occurring target cells may be converted to cells of relatively normal appearance by suspending them in the patient's own plasma rendered hypotonic with distilled water. The target cell is considered to be a cell whose envelope is large in relation to the contents regardless of the cause of this relationship. In the Mediterranean syndromes some evidences point to the possibility that the structural defects of the target cells are connected with faulty iron metabolism.

tions have been obtained. In Table IV are shown the results of such investigations. These substances may be employed systematically as a diagnostic procedure, thus constituting a "differential fragility test"[48] which is based on the concept that pathologic cells, when injured by different substances, react differently.

TABLE IV. DIFFERENTIAL FRAGILITY TESTS

SUBSTANCE	FINDINGS
Hypotonic saline solutions	Increased in conditions with spherocytosis, such as: Hereditary and acquired hemolytic jaundice Leucoerythroblastic anemia Decreased in conditions with leptocytosis (target cells) such as: Sickle-cell anemia Mediterranean syndromes Postsplenectomy state Severe liver disease Hemorrhage
Lysolecithin (Singer[16, 40])	Same as with hypotonic saline. Exceptions: blackwater fever[49]—increased lysolecithin fragility but normal saline fragility Acquired hemolytic anemia—normal lysolecithin fragility but increased saline fragility
Saponin (Erlsbacher and Kindermann[50])	Increased in pernicious anemia
Dilute hydrochloric acid (Ham[51])	Increased in Marchiafava-Micheli syndrome

Recently Hegglin and Maier[52] demonstrated a diminished "heat resistance" of the erythrocytes in the Marchiafava-Micheli syndrome. When blood is placed in an incubator at 37° C. for six hours, gross hemolysis of the clotted blood is considered specific evidence for the presence of this disorder. According to Minot and Castle,[53] this test does not depend on the heat resistance of the erythrocytes; it is caused by the accelerated acid production at the increased temperature of the incubator. Addition of alkali to the blood inhibits the effect of incubation. Doubtless other substances may be found and used in the future for the performance of the differential fragility test.

Mechanical fragility has also been studied and it was found that pathologic cells break up more easily than do normal ones when exposed to mechanical injury. All these findings again demonstrate that structural alterations of the red cells may also be detected by physicochemical methods.

As can be seen from these discussions, the many factors which may cause pathologic hemolysis seem always to be qualitatively different from those responsible for the physiologic disintegration of the red cells. The useful concept applied in other fields of medicine to explain pathologic phenomena as quantitative deviations from the normal, does not seem to be expedient in the hemolytic syndromes. However, if aging of the cell means physicochemical changes in the structure of the erythrocyte, then physiologic and pathologic disintegration could be correlated in the future when a better knowledge of the various characteristics of cell alterations may be available.

SUMMARY

There are three interdependent factors active in maintaining the normal red cell count: erythrocyte production, disintegration, and the life span of the red cell in the circulation. Shortening of the lifetime may be due to endogenous factors (i.e., faulty structure of the erythrocyte, abnormal hemolyzing activities), or may be caused by removal of erythrocytes from the circulation (hemorrhage).

With different methods, the true average lifetime was found to be from 100 to 120 days in dogs, monkeys, and man. Under strictly physiologic conditions, the disappearance of the red cells depends on their age. If pathologic mechanisms are at work, they concur with the physiologic processes and destroy the erythrocytes at random, irrespective of their age. The method of cross-determination of the lifetime of the erythrocytes (i.e., the survival time of normal cells in a patient with a hematologic disorder and of this patient's erythrocytes in a normal individual) demonstrates whether the cause of a disorder is due to an extra- or an intracorpuscular abnormality.

The lifetime of the red cells before and after splenectomy in dogs was found to be practically identical. After splenectomy the red cell population contained numerous target cells with increased resistance to hypotonic saline solutions; the longevity of these abnormally shaped cells is not prolonged.

Participation of the spleen in physiologic blood destruction seems to be absent or negligible. In certain hemolytic anemias (familial spherocytic jaundice, acquired hemolytic anemia) splenic hemolysis is present; it is selective, conditioned by abnormal properties of the erythrocytes which circulate through the organ. No evidence is available for the existence of a primary hemolytic "hypersplenism." The diminution of the pigment excretion observed after splenectomy in human beings is not indicative of a decrease of blood destruction.

The hemolytic syndromes may be classified in two main groups: (1) disorders due to a variety of substances which, by acting on the red cells, injure their structure, and (2) diseases in which there are various and characteristic structural defects present in the red cells at the start of their life. The physicochemical changes responsible for the different structural alterations are unknown. Defective cells usually, but not always, have a shortened life span. Some defects make cells vulnerable to physiologic mechanisms.

Structural defects manifest themselves morphologically by changes in size, shape, and volume of the red cells, or functionally in such a way that pathologic cells, when injured by different substances, react differently (differential fragility). The many factors which cause pathologic hemolysis seem always to be qualitatively different from those responsible for the physiologic disintegration of the erythrocytes. However, if aging of the cell means physicochemical change in its structure, then physiologic and pathologic disintegration could be correlated in the future when a better knowledge of the characteristics of the various cell alterations may become available.

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LABORATORY METHODS

A SIMPLE QUANTITATIVE TEST FOR THE PENICILLIN SENSITIVITY OF BACTERIA: THE "RADIAL STREAK" METHOD

FREDERIC E. MOHS, M.D.
MADISON, WIS.

WHEN penicillin therapy of an infected lesion is contemplated, it is important to determine, as soon as possible, whether or not the causative organisms are sensitive to the drug. If they are sensitive it is also advantageous to know the degree of sensitivity. A number of sensitivity tests have been devised, some of which are fairly simple.[1, 2] An even more simple quantitative test and one having several other advantages is provided by the "radial streak" method described herewith. This test is based on the original observations of Fleming[3] which indicated that penicillin diffuses through agar, preventing the growth of sensitive organisms for a distance proportional to the susceptibility of the organisms to the drug. In fact, the radial streak test is simply a modification of methods devised by Fleming.

MATERIALS AND METHODS

The only material required for the test is a 5 per cent blood agar plate, a disk of penicillin-impregnated gauze or filter paper, and a culture of *Staphylococcus aureus* of known sensitivity to penicillin. On the bottom of the Petri plate is drawn a central circle 15 mm. in diameter with radial lines extending to the edge of the plate, dividing it into six sectors. A stencil to guide the wax pencil used in marking is convenient.

Material directly from lesions or from cultures of the causative organisms are streaked on the blood agar by means of small cotton-tipped applicators. Two or three streaks extending from the edge of the central circle to the edge of the plate are made, keeping well within one sector (Fig. 1). An attempt should be made to use approximately the same amount of infected material for all of the streaks because the number of organisms has a slight effect on the degree of inhibition. It is sometimes impossible to judge accurately the number of organisms in the test material, especially when pus directly from a lesion is being tested. Under these circumstances, varying quantities of the material are used in the two or three streaks made in one sector. If, despite these precautions, the seeding is either too heavy or too light, it is desirable to repeat the test so that a more accurate reading may be obtained. When twenty-four hour cultures of the test organisms are used, this source of inaccuracy is minimal.

In a busy clinic a number of sensitivity tests are usually desired each day so the plate is returned to the refrigerator after each streaking until five sectors have been inoculated. For a control the sixth sector is streaked with a twenty-four-hour broth culture of *Staph. aureus* culture of known sensitivity to penicillin.*

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From the Chemosurgery Clinic, Wisconsin General Hospital, and the McArdle Memorial Laboratory, University of Wisconsin Medical School.

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*For uniformity, *Staphylococcus aureus*, strain 209-P, has been adopted as the control culture. This was obtained from the Food and Drug Administration through the kindness of Dr. Albert C. Hunter.

A penicillin-impregnated disk 15 mm. in diameter is then placed on the surface of the culture medium over the central circle. In the Chemosurgery Clinic penicillin ointment-impregnated gauze is kept on hand for the local treatment of infections as described elsewhere,[4] and it is convenient to use this material for the disk. This penicillin gauze is impregnated with a standard amount of an ointment containing 500 Oxford units per gram so the penicillin content of the disk is essentially constant. If the penicillin gauze is not available it is just as satisfactory to place a 15 mm. disk of filter paper on the culture plate and to drop upon it from a No. 20 hypodermic needle one drop of a solution containing 5,000 units per cubic centimeter.

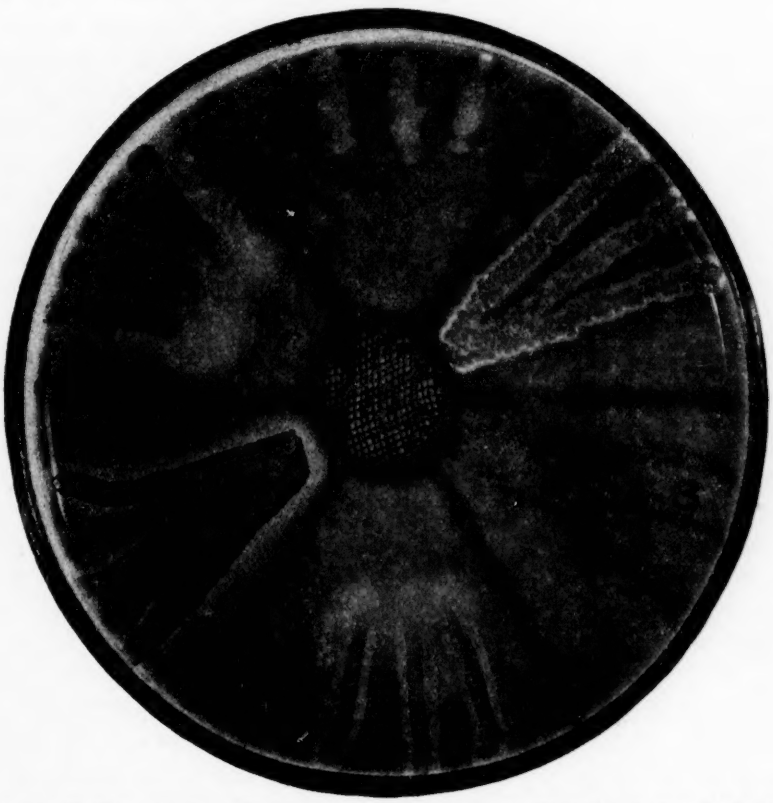

Fig. 1.—Radial streak culture plate showing the sensitivities of five test organisms (sectors *1* to *5*) in relation to the sensitivity of the control culture of *Staphylococcus aureus*, strain 209-P (sector *6*). Taking the 18 mm. of inhibition shown by the control as 100 per cent, the sensitivities of the test organisms are expressed as follows: sector *1* (*Streptococcus hemolyticus*), 21 mm. or 117 per cent; sector *2* (*Bacillus coli*), 0 mm. or 0 per cent; sector *3* (*Bacillus pyocyaneus*), 0 mm. or 0 per cent; sector *4* (*Staphylococcus aureus* from a boil which responded rapidly to local penicillin therapy), 18 mm. or 100 per cent; sector *5* (*Staphylococcus aureus* from a folliculitis which responded poorly to local penicillin therapy), 3 mm. or 17 per cent.

The culture plate is then placed in an incubator at 37° C. Usually the cultures are incubated overnight and the zone of inhibition is measured the next day. However, if a reading is wanted sooner, there is usually enough growth by eight hours for a fairly accurate determination of sensitivity.

The distance between the edge of the central circle and the uninhibited portion of the streak is measured with calipers. Taking the inhibition distance exhibited by the control organisms as 100 per cent, the relative sensitivities of the test organisms are expressed in per cent. Thus, if the control organism is inhibited for 13 mm. and the test organism is also inhibited for 13 mm., the sensitivity of the latter is 100 per cent. However, if the test organism is in-

hibited for 6 mm., its sensitivity is 46 per cent ($6 \div 13 \times 100$), while if it is inhibited for 21 mm., its sensitivity is 154 per cent ($21 \div 13 \times 100$).

DISCUSSION

Besides giving a quantitative determination of penicillin sensitivity, the radial streak culture also facilitates the identification of the causative organisms. Some bacteria produce characteristic colonies as, for example, *Staph. aureus*, yellow colony with or without hemolysis; *Bacillus pyocyaneus*, blue-green colony; *Streptococcus hemolyticus*, small grayish colony with variable degrees of hemolysis; and *Streptococcus viridans*, green color around colonies. In mixed infections various types of colonies may be selected for further studies by smears and cultures. Differing penicillin sensitivities as shown by Fleming[3] often aid in the separation and identification of the pathogens; for example, a penicillin-resistant organism which would ordinarily be overgrown by a predominating penicillin-sensitive organism will be found in the zone near the penicillin.

Besides being useful in connection with the various surgical specialties, the radial streak test may well have uses in dermatology, medical and pediatric clinics, and even in general practice. The plates may be carried in the physician's bag and inoculated in the patient's home; if smears and further cultures are desired they may be taken from the plate after incubation.

Other advantages of the radial streak test may be mentioned. The economy of time and material occasioned by the use of one plate for five lesions is worth while. The simultaneous inoculation of a control culture of known sensitivity gives reliable determinations without dependence upon standard penicillin solutions which may deteriorate. The exact amount of penicillin in the central disk is less important than in most sensitivity tests because the sensitivities of the unknown organisms are expressed in relation to the sensitivity of the control culture. The facility with which inoculation can be made of material directly from infected lesions permits the determination of sensitivity with minimal delay.

CONCLUSION

Testing for the penicillin sensitivity of bacteria is facilitated by the "radial streak" method. The only material needed is a blood agar plate, a penicillin-impregnated disk of gauze or filter paper, and a culture of *Staph. aureus* of known sensitivity.

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DISTILLING APPARATUS FOR CLINICAL LABORATORIES

O. H. GAEBLER, PH.D., M.D.
DETROIT, MICH.

DISTILLING apparatus of the all-glass type is essential in many procedures and more convenient than ordinary distilling apparatus in other procedures. Among instances that may be cited are preparation of constant boiling hydrochloric acid for standards, purification of hydrochloric and nitric acids for determination of lead and other metals, preparation of conductivity water or water free of metals, purification of alcohol and other organic solvents for use in lipid determinations, recovery of organic solvents, and determinations of alcohol or iodine in blood. The outfits described in this paper have been so designed as to minimize the amount of desk space required, to eliminate waste of time spent in assembling, and to reduce the cost of replacing broken parts. In constructing them, standard Pyrex parts listed in the catalogs of the firms supplying laboratory glassware have been used. The catalog names of parts are used in the following description.

ALL-GLASS APPARATUS

A one-piece outfit is shown in Fig. 1. The flask, which may be of any size from 500 to 3,000 c.c., is made from an ordinary Pyrex distilling flask by altering the neck and side arm. Distilling flasks with these parts broken can be used. A tube with outside diameter of 10 mm. is sealed into the neck and ends from 2 to 3 mm. above the bottom of the flask. The three-way stopcock has side arms with an outside diameter of 10 mm., and the bore of the plug is 4 mm. The unattached side arm is cut off 3.5 cm. from the shell of the stopcock and extends back at right angles to a plane passing through the axes of flask and condenser. The flanged cylindrical funnel is made from a test tube or from tubing. It is from 9 to 10 cm. long and has an outside diameter of 32 mm. The trap is a Kjeldahl connecting bulb of the Iowa State type, selected because of its sturdiness, efficiency, and low cost. Tubing attached to it should be of similar strength. The condenser is of the West improved type, and the inner tube may be either plain, as shown, or of the indented type. The dimensions given in Fig. 1 are for the 500 c.c. outfit. In the 3 liter outfit the height from the bottom of the flask to the top of the funnel is 57 cm., the delivery tip is 6 cm. below the level of the bottom of the flask, the distance between the neck of the flask and the condenser is 15 cm. instead of 12.5 cm., and the condenser has a 500 mm. jacket.

All sizes of this outfit up to 3 liters are satisfactorily supported by a single large castaloy extension clamp. The flask may be filled through the funnel, or water to be distilled may be siphoned in through the side arm of the stopcock. This side arm also serves for applying suction to empty the flask or to clean the condenser and flask by reverse washing. Insoluble residues can be removed by admitting hot chromic-sulfuric acid mixture through the funnel. The mixture is subsequently diluted and removed by suction. The outfit can be filled, emptied, and cleaned without removing it from the stand. Besides the purposes already mentioned, the tube within the flask serves to prevent bumping,

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since it becomes a boiling tube when the upper end is closed by the proper turn of the stopcock. The principle of preventing spray from passing over by means of two inclined tubes and an intervening trap is the same as in the apparatus designed by Yoe.

The 3 liter size has been routinely used for over five years in preparation of conductivity water and constant boiling hydrochloric acid, or for purification of acids. By redistilling ordinary distilled water alone or after addition of alkali and permanganate, water with conductivity of 1×10^{-6} is regularly obtained if the first fourth of the distillate is discarded. The 500 c.c. size has been used in determination of alcohol or iodine in blood. In the latter determination a trace of chromium is carried over, since the concentration of chromic salt in the

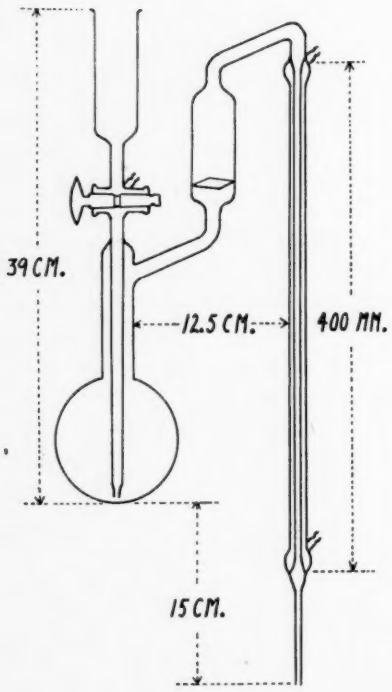

Fig. 1.

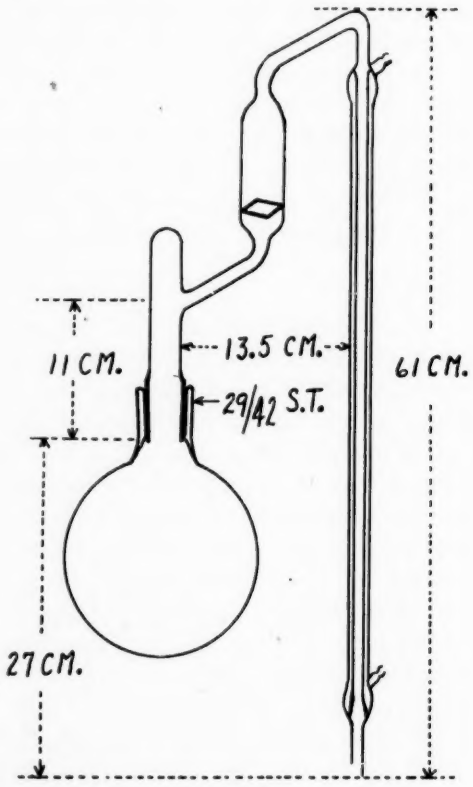

Fig. 2.

flask is very great. Interference of chromate in the final titration of iodate can, however, be prevented.* Vertical outfits like those shown in Figs. 1 and 2 occupy only a twelve- to fifteen-inch square of desk space. The disadvantage that the outlet of the condenser is near the flame is easily overcome by using a burner shield, or, in the case of inflammable liquids, an adapter which will convey the condensed liquid to a receiver on the floor. The adapter is connected to the condenser with a one-hole stopper with which the distillate does not come in contact.

In designing the two-piece outfit shown in Fig. 2, advantage was taken of the fact that Pyrex round-bottom and flat-bottom flasks of all capacities from 1 to 3 liters are regularly supplied with the same size of standard taper joint.

*Gaebler, O. H., and Baty, M.: Detection and Titration of Chromate in Blood Iodine Determinations, Indust. Engin. & Chem. (Anal. Ed.) 13: 442, 1941.

Of the 1 liter size, the type with a long neck should be used. The other part shown in Fig. 2 is made from the inner cone of a 29/42 standard taper joint, a Kjeldahl connecting bulb, and a West improved type condenser. The inlet and outlet of the condenser extend back at right angles from the plane of the apparatus. As shown in Fig. 2, the inner cone of the standard taper joint has been sealed off at the upper end. A dropping funnel or other equipment can readily be sealed in at this point if desired.

Reference to catalogs will show that the standard flasks used in the present outfit cost only a fourth as much as special flasks. The cost of all parts indicates that the apparatus shown in Fig. 2, with three flasks of different sizes, should be less expensive than another outfit which has only one flask and no trap.

MICRO-KJELDAHL DISTILLING APPARATUS

For class use, an inexpensive type of apparatus which will not suck back when the flame blows about is required. The design shown in Fig. 3 meets these requirements. When the amount of nitrogen is between 1.0 and 5.0 mg., results are so precise that the expensive forms of apparatus advertised for this simple procedure seem unjustified.

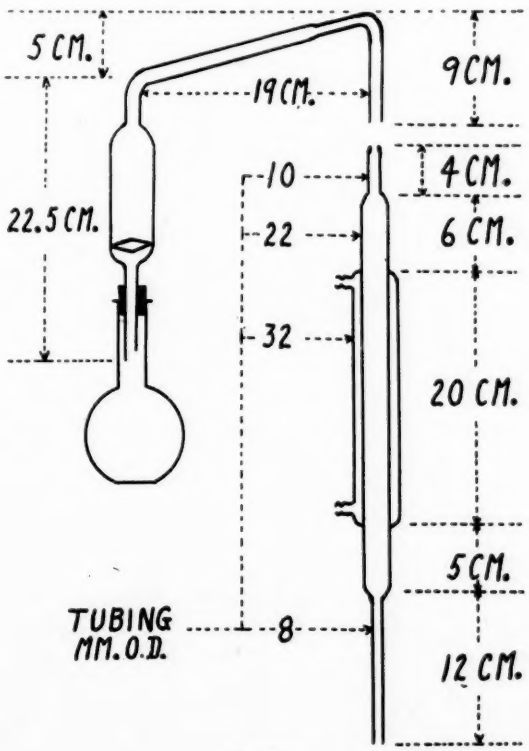

Fig. 3.

The flask is a 250 c.c. Pyrex boiling flask. A No. 4 pure gum amber, or other alkali-resisting type of stopper, must be used. The Kjeldahl connecting bulb has been altered by bending the upper tube to the indicated angle and sealing on a piece of 10 mm. tubing. The latter is bent downward to connect with a condenser through a piece of rubber tubing not shown in Fig. 3. The capacity of the inner tube of the condenser exceeds the usual volume of acid or distillate present above the delivery tip; hence the solution in the receiver can not suck

back. The 4 cm. tip attached to the upper end of the inner tube of the condenser is thickened at its free end to increase its strength and is constricted slightly to facilitate slipping the rubber tubing on and off.

The stopper and that part of the neck of the flask which it enters should be free of alkali and water when the connection is made. The flask is best heated directly, for if a screen is used the heat passing around it converges upon the stopper and soon ruins it. Distillation is stopped by grasping the cold water jacket of the condenser with one hand and working off the rubber connection from the upper end of the condenser with the other.

SUMMARY

Distilling apparatus is described which is suitable for the following purposes: preparation of conductivity water, of constant boiling hydrochloric acid, and of water and acids free of lead and other metals, purification or recovery of organic solvents, determination of blood alcohol or blood iodine, and micro-Kjeldahl distillations.

BOOK REVIEWS

The Bacterial Cell in Its Relation to Problems of Virulence, Immunity and Chemotherapy. Harvard University Monograph in Medicine and Public Health No. 6. By *Rene J. Dubos,* recent Professor of Comparative Pathology and Tropical Medicine, Harvard Schools of Medicine and Public Health, and present member of the Rockefeller Institute. Harvard University Press, Cambridge, Mass. Price $5.00. Cloth with 460 pages.

This excellent volume may well prove to be an important catalyst for stimulating the further development of bacteriology as a biologic science in its own right, as distinguished from its obvious and important utilitarian aims in the fields of medicine, industry, and agriculture.

Due to their microscopic size and absence of recognized sexual mechanism of reproduction, there has arisen an illusion that bacteria possess very primitive simple organization. Actually bacteria perform an amazing multiplicity of functions, and it appears very likely that the failure to recognize in them the corresponding multiplicity of cellular structures underlying these functions is an indication of the deficiency of our techniques rather than an evidence of the simplicity of their organization.

The basic aim of the author is to bring together and to correlate information, which has accumulated as result of various investigations in the fields of bacteriology and immunology, in so far as it tends toward eliciting the "biochemical architecture" of the bacterial cell, and to integrate this information with that secured from the conventional study of bacterial structures by means of cytological techniques.

Inasmuch as this book represents an outgrowth of a course of eight lectures delivered by the author in February, 1944, under the auspices of the Lowell Institute in Boston, the basic contents are presented in eight chapters.

In the first chapter, entitled Materials, Problems and Methods, the author emphasizes the questionable validity of any attempt at reconstruction of the trends and direction of evolution in any group of living organisms on morphological grounds alone. It is particularly true of such organisms as bacteria which not only possess very few morphological characteristics useful in classification, but in addition exhibit a high degree of mutability.

In the second chapter, entitled Cytology of Bacteria, are discussed data concerning the cell structures as elicited by microscopic examination with visible and ultraviolet light, as well as newer data secured by electronic microscopy.

The third chapter describes Physicochemical and Staining Properties of Bacteria with the purpose of eliciting information as to composition and distribution within the cell of various reacting constituents.

The fourth chapter consists of Analysis of Cellular Structure by Biochemical and Biological Methods which contributes information as to structure of the bacterial cell. In this connection the author stresses the role of bacterial enzymes and of serological methods as tools for microanalysis. The strict specificity and great reactivity of the later reagents, especially, permits not only the detection of small amounts of specific cellular constituents in the presence of relatively large quantities of contaminating substances, but, in many instances, they also give information concerning the relative position of these components.

In the fifth chapter, Variability of Bacteria, the author emphasizes the extraordinary ease with which bacteria adapt themselves to the environment either by reversible modification or by means of hereditary variation. This plasticity makes them unique as material for the study of certain problems of genetics, which cannot be investigated with the use of other physiologically more rigid material.

In the sixth chapter, devoted to the discussion of the Nature of Virulence, the author emphasizes that virulence is the result of a summation of a number of independent attributes which determine the ability of the parasite to establish itself in the host. Loss of any one of these component factors of virulence, as result of variation, tends to diminish or destroy the ability of the parasite to establish a pathological state.

In Chapter VII, entitled Immunization Against Bacterial Infection, the author attempts to analyze the present relatively limited information concerning the "protective antigens," i.e., those individual components of the bacterial cell which induce the production of antibodies upon which depends resistance to infection. He brings together the evidence which suggests that the location and orientation of these different antigenic components in the bacterial cell are important in determining the protective value of the antibodies to which they give rise.

In Chapter VIII the author discusses Bacteriostatic and Bactericidal Agents. In this discussion he considers not only the role of specific inhibitors of essential metabolic reactions, but also the enzymic and chemical reagents which can selectively alter or destroy vital morphological structures, as well as the nonspecific physicochemical reactions which affect the protoplasmic constituents of all living cells.

In the ninth chapter, Trends and Perspectives, the author attempts to reconstruct the trend of "bacteriological philosophy" during the present century. Much of the theoretical knowledge of the field of bacteriology and immunology was gathered as a by-product of the efforts to solve practical problems by empirical methods. Frequently, successful practices were developed before there was any understanding of their rational basis. Indeed, it was the analysis of the mode of action of these empirical methods which led to the recognition of a number of reactions between bacteria and the environment and to the subsequent description of the components of the cell involved in these reactions. The author suggests that in order to bring bacteriology to the state of development achieved by other sciences, it is necessary to abandon this anthropocentric attitude which characterized its earlier development—bacteria must be studied not only in the effects which they have on practical human problems, but also for what they are and what they do as independent living organisms.

Since the purpose of the book is to bring together the knowledge of cellular organization, the author appropriately invited Dr. C. F. Robinson to contribute a chapter on Nuclear Apparatus and Cell Structure of Rod-Shaped Bacteria, which reviews the factors which have delayed recognition of morphological organization of bacterial cells and describes some of the newer findings made possible through the employment of newer cytological procedures.

Although the text is replete with technical information, which will be of especial interest to bacteriologists and immunologists, the author has supplied the necessary background so skillfully that even the most technical portions of the text will present no difficulty for biologists outside of the special field of bacteriology.

J. BRONFENBRENNER.